PATENT ABSTRACTS OF JAPAN

(11)Publication number : 2003-274963
(43)Date of publication of application : 30.09.2003

(51)Int.Cl. C12N 15/09
A61M 1/36
C12N 5/10
C12Q 1/02
// A61K 35/14
A61P 1/16
A61P 7/08
A61P 43/00

(21)Application number : 2002-081344

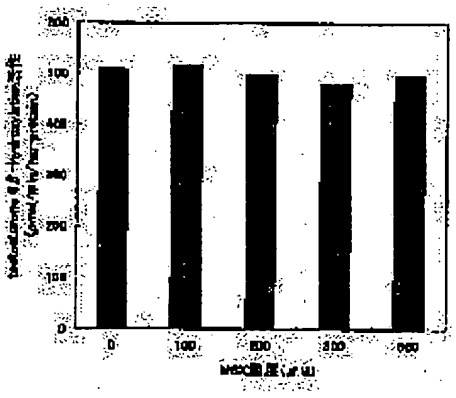
(22)Date of filing : 22.03.2002

(71)Applicant : JAPAN SCIENCE & TECHNOLOGY CORP
NATIONAL CENTER FOR CHILD HEALTH & DEVELOPMENT

(72)Inventor : OMASA TAKESHI
SUGA KENICHI
KISHIMOTO MICHIMASA
KATAKURA HIROO
ENOSAWA SHIN
TSURUOKA SHUICHI

(54) GENE RECOMBINANT CELL STRAIN AND LIVER FUNCTION SUPPORTING EQUIPMENT USING THE SAME

(57)Abstract:
PROBLEM TO BE SOLVED: To obtain a cell strain which is transformed with a drug metabolism enzyme gene and an ammonia metabolism enzyme gene such as glutamine synthase gene and has drug metabolism ability and a drug metabolism assay system which evaluates the function of the cell strain and uses the cell strain and to provide liver function supporting equipment such as a hybrid type artificial liver, etc., selectively removing toxic substances.
SOLUTION: The cell strain is transformed with the drug metabolism enzyme gene such as P450 3A4, etc., and the ammonia metabolism enzyme gene such as the glutamine synthase gene, etc., especially a human liver-derived cell strain such as HepG2 cell strain. The liver function supporting equipment such as the hybrid type artificial liver, etc., is obtained by using the cell strain. The drug metabolism assay system is obtained by using the cell strain.



LEGAL STATUS
Date of request for examination] 13.08.2004

Date of sending the examiner's decision of rejection]
Kind of final disposal of application other than the

examiner's decision of rejection or application converted registration]

[Date of final disposal for application]

[Patent number]

[Date of registration]

[Number of appeal against examiner's decision of rejection]

[Date of requesting appeal against examiner's decision of rejection]

[Date of extinction of right]

* NOTICES *

CLAIMS

[Claim(s)]
[Claim 1] The cell strain in which the transformation was carried out by the drug-metabolizing enzyme gene and the ammonia metabolic turnover enzyme gene.
[Claim 2] The cell strain according to claim 1 whose drug-metabolizing enzyme gene is P450.
[Claim 3] Drug-metabolizing enzyme is P450. Cell strain according to claim 2 which is 3 A4.
[Claim 4] The cell strain of claim 1-3 given in any 1 term a given ammonia metabolic turnover enzyme gene is a glutamine synthetase gene.
[Claim 5] The cell strain of claim 1-4 given in any 1 term a given cell is the mammals animal origin.
[Claim 6] The cell strain according to claim 5 whose cell is the Homo sapiens liver origin.
[Claim 7] The cell strain according to claim 6 whose cell is the Homo sapiens hepatocyte origin.
[Claim 8] The cell strain according to claim 7 whose cell is HepG2.
[Claim 9] The cell strain of claim 1-8 given in any 1 term a given cell is a transgenics ammonia metabolic turnover Homo sapiens hepatocyte stock.
[Claim 10] The cell strain of claim claim 1-8 characterized by introducing the drug-metabolizing enzyme gene and the ammonia metabolic turnover enzyme gene into the common expression vector given in any 1 term.
[Claim 11] The cell strain according to claim 10 whose expression vector is a plasmid.
[Claim 12] The cell strain according to claim 10 or 11 which is the plasmid which is the mammals cell expression vector in which an expression vector has two or more independent multi-cloning sites.
[Claim 13] The cell strain according to claim 12 which is an expression vector pBudCE4.
[Claim 14] A cell strain given in claim 1 thru/or any 1 term of 13 characterized by showing the P450 activity of about 200 or more pmol/min per protein mg.
[Claim 15] The cell strain according to claim 14 characterized by showing the P450 activity of about 490 or more pmol/min per protein mg.
[Claim 16] The cell strain according to claim 14 or 15 characterized by maintaining the P450 activity of about 420 pmol/min for 80 days per protein mg.
[Claim 17] The liver function auxiliary device which contains the cell strain of a publication in claim 1 thru/or any 1 term of 16.
[Claim 18] Furthermore, a cell strain given in claim 1 thru/or any 1 term of 17 by which the transformation is carried out with another drug-metabolizing enzyme gene.
[Claim 19] The liver function auxiliary device which uses the cell strain of a publication for claim 1 thru/or any 1 term of 17.
[Claim 20] Furthermore, the liver function auxiliary device containing a cell strain according to claim 19 which uses the cell strain of another kind.
[Claim 21] The liver function auxiliary device according to claim 20 whose cell strain of another kind is the Homo sapiens nonparenchymatous liver cell origin.
[Claim 22] The liver function auxiliary device according to claim 20 or 21 which is a hybrid mold artificial liver.
[Claim 23] A liver function auxiliary device given in claim 20 thru/or any 1 term of 22 containing a time style type culture apparatus.
[Claim 24] The drug metabolism assay system which uses the cell strain of a publication for claim 1 thru/or any 1 term of 17.
[Claim 25] a) Cultivate the cell strain of a publication in claim 1 thru/or any 1 term of 17, add b measuring object matter to a culture medium, and carry out predetermined period culture further. c) Extract a supernatant, measure

the concentration of the measuring object matter in the supernatant of which d extraction was done, and the metabolized measuring object matter, respectively, and it asks for both ratio of concentration. e) The measuring object matter and the examined substance of suitable concentration are added to a culture medium, predetermined period culture is carried out further, and it is the f above-mentioned step c. It reaches. d Drug metabolism assay system which consists of measuring the effectiveness exerted on the metabolic turnover of the measuring object matter by the examined substance from change of g ratio of concentration repeatedly.

[Claim 26] a) Cultivate the cell strain of a publication in claim 1 thru/or any 1 term of 17, add b nifedipine to a culture medium, and carry out predetermined period culture further. c) Extract a supernatant, measure the concentration of the nifedipine in the supernatant of which d extraction was done, and oxidization mold nifedipine, respectively, and it asks for both ratio of concentration. e) Nifedipine and the examined substance of suitable concentration are added to a culture medium, predetermined period culture is carried out further, and it is the f above-mentioned step c. It reaches. d Drug metabolism assay system which consists of measuring the effectiveness exerted on the oxidization mold nifedipine production by the examined substance from change of g ratio of concentration repeatedly.

[Claim 27] a) Cultivate the cell strain of a publication in claim 1 thru/or any 1 term of 17, add b midazolam to a culture medium, and carry out predetermined period culture further. c) Extract a supernatant, measure the concentration of the midazolam in the supernatant of which d extraction was done, and oxidation type midazolam, respectively, and it asks for both ratio of concentration. e) Midazolam and the examined substance of suitable concentration are added to a culture medium, predetermined period culture is carried out further, and it is the f above-mentioned step c. It reaches. d Drug metabolism assay system which consists of measuring the effectiveness exerted on the oxidation type midazolam production by the examined substance from change of g ratio of concentration repeatedly.

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] This invention relates to the liver function auxiliary device which uses the cell strain in which the transformation was carried out by the drug-metabolizing enzyme gene and the ammonia metabolic turnover enzyme gene, and this cell strain.
[0002]
[Description of the Prior Art] Many [in our country / acute liver failure patients including viral hepatitis] Acute liver failure is **** by a rapid hepatocyte necrosis or a rapid hepatocyte malfunction about hepatic encephalopathy, icterus, ascites, a bleeding tendency, renal failure, etc. Liver function adjuvant therapy which combined hemofiltration dialysis (continuous hemodiafiltration continuous arteriovenous hemofiltration:CHDF) and plasma exchange (plasma exchange-E) is enforced at the current clinical place to these. This is the approach of filtering from blood the matter which should be counteracted [the poison] for which and metabolized by liver, or removing with plasma, and filling up an insufficiency with Homo sapiens normal plasma. However, 1, 2, and 3 which have come to obtain sufficient treatment results since the compensation of all the liver functions covering a variety cannot be carried out. A liver transplantation is 4 which is a problem with the very serious lack of a donor although it is the cure by which acute liver failure was established. Urgent correspondence until a donor liver is transplanted (bridge use) (development of the liver function auxiliary device with which the liver function of the ving body which did and lapsed into liver failure can be assisted is an important technical problem.) Moreover, hepatocyte is 5 which has the very flourishing ability to regenerate. A period until the liver which lapsed into acute ver failure is reproduced and it recovers a function also from such a field, and the importance of assisting a liver unction using a liver function auxiliary device are 6 [high].
0003] A hybrid mold artificial liver (biotechnology artificial liver) is mentioned as one of the basis of such a background, and the liver function auxiliary devices. 7 which combines an animal cell (living thing-ingredient) with a hybrid mold artificial liver at a bioreactor (physical ingredient). 8-14 by which various devices, such as the circulation approach of of the restoration approach and blood of the structure and the animal cell of a bioreactor, re made in order to raise the function as an artificial liver.
0004]
[Problem(s) to be Solved by the Invention] The animal cell used for a hybrid mold artificial liver is roughly classified into two. One uses the separation hepatocyte of the heterozoic origins, such as Buta. Although these have the high liver function, a liver function is unmaintainable for a long period of time except that cell supply takes time and effort and time amount. Moreover, since it is heterozoic, there is danger, such as immunorejection and strange virus infection. Then, what is used in the form where face to face is stood against these is HepG2. Including, it is a Homo sapiens origin cell strain. Although a liver function is low, there is a property maintainable over a long period of time that cell supply is easy. Then, we considered making some of liver functions give this Homo sapiens origin cell strain.
0005] this invention persons are the Homo sapiens liver origin cell strains HepG2 until now. 15 which succeeded in making the removal ability of the ammonia which is one of the toxic substances give a cell. Then, by making the capacity to metabolize drugs considered to be important for a degree as a function of a biotechnology artificial liver give Homo sapiens liver origin cell strain HepG2 cell, I thought that he wanted to build the biotechnology artificial liver as an alternative removal system of a toxic substance. Therefore, construction of HepG2 cell strain which has capacity to metabolize drugs, and its functional evaluation are our purposes. Moreover, the use as a model (models, such as a toxicity test of a drug and specification of a metabolic fate) of drug metabolism research

in / besides the liver failure therapy purpose / in the animal cell which has these capacity to metabolize drugs / a Homo sapiens liver] is also considered.

0006]

Means for Solving the Problem] By the way, a drug metabolism reaction is 16 divided roughly into the first phase reaction and the second phase reaction. Polar groups, such as a hydroxyl group, a carboxyl group, and an amino group, generate the first phase reaction by oxidization, reduction, hydrolysis, etc., or the thing of the reaction introduced is said. Although these functional groups are comparatively small polar groups compared with the second phase reaction, generally a drug loses the compatibility over a site of action by these polarization. Consequently, it becomes the form which is easy to be excreted while a pharmacological action or a physiological function falls. The second phase reaction is a reaction into which a bigger substituent than the first phase reaction is introduced. It is the conjugation reaction into which glucuronic acid, a sulfuric acid, some amino acid, a glutathione, etc. are introduced. Although there are not few compounds which have beforehand functional groups, such as a hydroxyl group, a carboxyl group, or an amino group, after these functional groups generate by the first phase reaction or being introduced, there are many compounds which undergo the second phase reaction. Since the substituent of a conjugation reaction has the polarity higher than the functional group introduced at the first phase reaction, it tends [further] to receive elimination, and it loses a pharmacological action or a physiological function.

0007] About 80% of the drug metabolism in Homo sapiens is the cytochrome P450 which exists in a hepatic microsome. It is bearing. These P450 is bearing the first phase reaction, and consists of various subfamilies. 17 which is said for the metabolic turnover capacity of the first phase reaction to decline especially in the fulminant hepatitis patient, and is said to be especially clinically important, to occupy about 30% of the amount of manifestations in an adult liver, and to metabolize the drug of varieties also in it, and 18 that -- P450 3 A4 it is . Then, this invention person is this P450 3 A4, when evaluating the capacity to metabolize drugs in an animal cell first. It decided to observe activity. Moreover, P450 3 A4 By building an expression vector and introducing this into an animal cell, it is P450 3 A4. It tried making it discovered by the animal cell. And the possibility of the clinical application as a cell both used for a biotechnology artificial liver which performs functional evaluation about the obtained cell strain was examined, and this invention was completed.

0008] That is, this invention relates to each following mode.

. Cell strain in which transformation was carried out by drug-metabolizing enzyme gene and ammonia metabolic urnover enzyme gene.

. Cell strain of one above-mentioned publication whose drug-metabolizing enzyme gene is P450.

. Drug-metabolizing enzyme is P450. Cell strain of two above-mentioned publication which is 3 A4.

. Cell strain of the above 1-3 given in any 1 term given ammonia metabolic turnover enzyme gene is glutamine ynthetase gene.

. Cell strain of the above 1-4 given in any 1 term given cell is the mammals animal origin.

. Cell strain of five above-mentioned publication whose cell is the Homo sapiens liver origin.

. Cell strain of six above-mentioned publication whose cell is the Homo sapiens hepatocyte origin.

. Cell strain of seven above-mentioned publication whose cell is HepG2.

. Cell strain of the above 1-8 given in any 1 term given cell is transgenics ammonia metabolic turnover Homo apiens hepatocyte stock.

0. The cell strain of the above-mentioned above 1-8 characterized by introducing the drug-metabolizing enzyme ene and the ammonia metabolic turnover enzyme gene into the common expression vector given in any 1 term.

1. The cell strain of ten above-mentioned publication whose expression vector is a plasmid.

2. The cell strain of the above 10 or 11 publications which are the mammals cell expression vectors in which an xpression vector has two or more independent multi-cloning sites and which are plasmids.

3. The cell strain of 12 above-mentioned publication which is an expression vector pBudCE4.

4. A cell strain given in the above 1 thru/or any 1 term of 13 characterized by showing the P450 activity of about 00 or more pmol/min per protein mg.

5. A cell strain given in the above 14 characterized by showing the P450 activity of about 490 or more pmol/min er protein mg.

6. A cell strain the above 14 characterized by maintaining the P450 activity of about 420 pmol/min for 80 days er protein mg, or given in 15.

7. The liver function auxiliary device which contains the cell strain of a publication in the above 1 thru/or any 1

:erm of 16.
18. A cell strain given in the above 1 thru/or any 1 term of 17 by which the transformation is carried out with still nore nearly another drug-metabolizing enzyme gene.
19. The liver function auxiliary device which uses the cell strain of a publication for the above 1 thru/or any 1 term of 17.
20. The liver function auxiliary device which furthermore uses the cell strain of another kind and which contains :he cell strain of a publication in the above 19.
21. The liver function auxiliary device of 20 above-mentioned publication whose cell strain of another kind is the Homo sapiens nonparenchymatous liver cell origin.
22. The liver function auxiliary device of the above 20 or 21 publications which are hybrid mold artificial livers.
23. A liver function auxiliary device given in the above 20 thru/or any 1 term of 22 containing a time style type culture apparatus.
24. The drug metabolism assay system which uses the cell strain of a publication for the above 1 thru/or any 1 :erm of 17.
25.
a) Cultivate a cell strain the above 1 thru/or given in any 1 term of 17, add b measuring object matter to a culture nedium, and carry out predetermined period culture further. c) Extract a supernatant, measure the concentration of the measuring object matter in the supernatant of which d extraction was done, and the metabolized measuring object matter, respectively, and it asks for both ratio of concentration. e) The measuring object matter and the examined substance of suitable concentration are added to a culture medium, predetermined period culture is carried out further, and it is the f above-mentioned step c. It reaches. d Drug metabolism assay system which consists of measuring the effectiveness exerted on the metabolic turnover of the measuring object matter by the examined substance from change of g ratio of concentration repeatedly.
26.
a) Cultivate a cell strain the above 1 thru/or given in any 1 term of 17, add b nifedipine to a culture medium, and carry out predetermined period culture further. c) Extract a supernatant, measure the concentration of the nifedipine in the supernatant of which d extraction was done, and oxidization mold nifedipine, respectively, and it asks for both ratio of concentration. e) Nifedipine and the examined substance of suitable concentration are added :o a culture medium, predetermined period culture is carried out further, and it is the f above-mentioned step c. It reaches. d Drug metabolism assay system which consists of measuring the effectiveness exerted on the oxidization mold nifedipine production by the examined substance from change of a ratio of concentration repeatedly.
27.
a) Cultivate a cell strain the above 1 thru/or given in any 1 term of 17, add b midazolam to a culture medium, and carry out predetermined period culture further. c) Extract a supernatant, measure the concentration of the nidazolam in the supernatant of which d extraction was done, and oxidation type midazolam, respectively, and it asks for both ratio of concentration. e) Midazolam and the examined substance of suitable concentration are added to a culture medium, predetermined period culture is carried out further, and it is the f above-mentioned step c. It reaches. d Drug metabolism assay system which consists of measuring the effectiveness exerted on the oxidation type midazolam production by the examined substance from change of b ratio of concentration repeatedly.
[0009] As drug-metabolizing enzyme, it is P450 in the thing of arbitration well-known to this contractor, for example, the various enzymes belonging to a cytochrome P450, and a concrete target. One sort chosen from 3 A4 CYP3 A4), CYP2C, CYP1A2, CYP2E1, CYP2D6, and CYP2A6 grade or two sorts or more can be used. In these, it s P450 3 A4. In an adult liver, the abbreviation one half of the drugs which occupy the amount of manifestations of about 30 %, and are used by clinical can be metabolized. This P450 3 A4 P450 2D6 which metabolizes many drugs next P450 2C It is thought important to also make it discovered. It is especially P450 2D6. It is related and s observed also from the field of genetic polymorphism. This P450 2D6 The patient (PM) without activity is mportant also from a viewpoint of development of the biotechnology artificial liver used not only for the way of hinking called development of the biotechnology artificial liver which it is called 5 - 6 % by the white with 0.8 % extent although it is few, and is used for an acute liver failure patient's therapy but for the therapy of such PM by Japanese people. As an example of an ammonia metabolic turnover enzyme gene, the glutamine synthetase gene of the CHO cell origin can be mentioned, for example. From the cDNA library of marketing or public engine

possession, or a vector, with cloning means, such as PCR, each of each of these genes can be easily come to hand or prepared, if it is this contractor. Moreover, the base sequence of these genes is indicated by various reference. As for the cell strain set as the object of a transformation, it is desirable that it is the mammals animal origin, for example, its cell strain of the Homo sapiens liver origin is more desirable, and it can mention HepG2 cell strain as an example of this cell strain. Such a cell can be received from various public engines (cell bank). Furthermore, it is the purposes, such as raising drug metabolism activity, and the cell strain obtained by also being able to carry out cloning of the cell strain of this invention obtained by carrying out a transformation, and carrying out cloning in this way is also the range of this invention. Although it may be supported by the different expression vector, respectively and a transformation may be performed separately, if the drug-metabolizing enzyme gene and the ammonia metabolic turnover enzyme gene are supported by the common expression vector, they are efficient and convenient. Although the vector of well-known arbitration can be used for this contractor as this expression vector, the mammals cell expression vector pBudCE4 which has two or more independent multi-cloning sites, for example, an expression vector, is suitable. In addition, the approach and means of well-known arbitration can perform easily each actuation of installation of each gene to an expression vector, the transformation of the cell strain by this expression vector, etc. by the technical field concerned. The transformation of the cell strain of this invention may be carried out with the drug-metabolizing enzyme gene of still more nearly another class. As an example of such drug-metabolizing enzyme, it is P450. The drug-metabolizing enzyme which bears not only the first phase reaction to depend but the second phase reaction can be mentioned. For example, since it is carried out by UDP-GT (UDP-glucuronyltransfera se), glucuronide conjugation is this UDP-GT. It is thought effective to introduce an expression vector further. Although the liver function auxiliary device of this invention can take the configuration of well-known arbitration to this contractor, the type of the hybrid mold artificial liver containing a time style type culture apparatus is suitable. In this equipment, although it is the translation which uses the cell strain of this invention as a living thing-ingredient, the cell of other classes can also be further used as a living thing-ingredient. The drug metabolism assay system of this invention can be characterized by using the above-mentioned cell strain, and can perform it by measuring the effect of the examined substance exerted on the metabolic reaction (for example, oxidation reaction, a hydroxylation reaction) of measuring object matter (standard substance), such as nifedipine and midazolam, according [for example,] to the cell strain.

[0010] Although it ** in the example and this invention is explained in full detail hereafter, the technical range of this invention is not **(ed) at all by these.

[0011]

[Example 1] The subjects of an experiment and an approach reagent used Wako Pure Chem or the reagent chemicals of Nakarai Tesuku, unless it mentioned especially. 1 . Animal Cell Culture Approach 1.1. Host Animal Cell HepG2 Origin (Institute of Physical and Chemical Research Cell Bank RCB0459) Human Hepatocellular Carcinoma Growth Gestalt Epithelial-like [0012] 1 A .2. animal cell culture culture-medium culture medium is 0.22. muM It was used after carrying out filtration sterilization using a membrane filter (Falcon;7105). - RDF RDF (Gln+) Medium composition RDF (Gln+) (HO) powder (Japanese-made medicine) 8.44 g glucose 2.58 gNaHCO 3 2.0 g glutamine 0.333 g penicillin G 58.8 mg streptomycin 120 mgMilli-Q Water In case 1 L notes 1 use is carried out, it is 10% (vol.%) considerable-amount ****** of culture-medium capacity about fetal calf serum (fetal bovine serum;(FBS) Gibco).

Notes 2 Zeocin (Invitrogen;R250-01) was added if needed. - RDF RDF (Gln-) () [Gln-] Medium composition RDF (HO) powder (Japanese-made medicine) 8.44 g glucose 2.58 gNaHCO 3 2.0 g glutamic acid 0.336 gNH 4 Cl 0.122 g penicillin G 58.8 mg streptomycin 120 mgMilli-Q Water 1 L notes 1 glutamic acid and NH 4 Cl A presentation RDF t determined to become the same number of mols as the glutamine of a culture medium (Gln+).

Notes 2) RDF The (HO) culture medium is the custom-made item of Japanese-made medicine, and is the usual RDF. A glucose and a glutamine are extracted from a culture medium.

Notes 3) FBS beforehand dialyzed when using it 10% (vol.%) considerable-amount ****** of culture-medium capacity.

Notes 4) It is MSX (MSX (Sigma;M-5379) was added according to each MSX concentration.) to a culture medium. When needed (high MSX it is used for the acquisition experiment of a resistant strain)

Notes 5) Accept the need and it is geneticin (and (Sigma (G418);G5013) Zeocin was added.) at the time of use.

- a blood serum -- medium composition NaHCO 3 0.2 g penicillin G for culture-medium blood serum culture for culture (1700 units/mg) 5.88 mg streptomycin The cellulose tube for 12 mgFBS 100 mL dialysis blood serum dialysis (Sanko Junyaku ;;Size 27/32) It dips in NaHCO 3 and 1 mMEDTA (pH 8.0) 2% (w/v), and is 10. After

carrying out the autoclave between parts, It cools to 4 **, a blood serum is put into this, and it is 10 of a blood serum. The dialysing fluid of the amount of double is used and it is 30. Between parts -> 1 Time amount -> 2 Time amount -> 3 Time amount -> 4 Time amount -> over night It dialyzed.
Blood serum dialysing fluid NaCl 8.0 gKCl 0.2 gNa 2 HPO 4 and 12H 2 O 2.9 gKH 2 PO 4 0.2 g kanamycin 32 mg streptomycin 120 mg distilled water 1 L [0013] 1 As Fundamental Matter in .1.3. Animal Cell Culture, and an Actuation 1:1.3.1. Cell Culture Container Cell Culture Container 3 T-flask of a class (Sumitomo Bakelite; -- MS-20050 (area-of-base 25 cm 2, capacity 50mL) --) MS-21250 (75 cm 2, 250 mL) and MS-20800 (225 cm2, 800 mL) (A following and small T-flask, an inside T-flask, a large T-flask and an abbreviation) or 100 mm dish (Corning;25020) was used. here -- a small T-flask and 100 mm dish **** -- usually -- 10 mL a culture medium -- an inside T-flask -- 30 mL a culture medium -- a large T-flask -- 90 mL The culture medium was put in. The small T-flask was used for the subculture of the usual cell strain. Moreover, by culture experiment, it is 100 mm dish. It used.
[0014] 1 .3.2. It is 1 in principle [of a cell / passage]. Every day (checking that a cell is in a confluent condition) it carried out.)
1) The old culture medium was attracted and removed with the pipet, and 0.25% trypsin solution of the following capacity was added.
Smallness T-flask [-- It is about 10 at 30 mL2 37 **. / It cultivated between parts.] -- 5 mL, inside T-flask -- 15 mL, large T-flask
3) if a cell peels and it becomes round -- a new culture medium -- the equivalent -- in addition, pipetting was improved (since the trypsin inhibitor is usually contained into the blood serum, the reaction which strips a cell stops at this time).
4) Move cell suspension to a centrifuging tube and it is 10 at 80 xg. After carrying out part at-long-intervals alignment separation, suction removal of the supernatant was carried out with the pipet.
5) The fresh culture medium was added in the centrifuging tube, the cell was suspended, and suitable amount inoculation was carried out at new T-flask.
[0015] 0.25% trypsin solution NaCl 8 gKCl 0.2 gNa 2 PO 4 and 12H 2 O 2.9 gKH 2 PO 4 0.2 gTRYPSIN 2.5 (Difco 1:250) gMilli-Q Water 1 L [0016] 1 .3.3. In the cryopreservation of the cryopreservation method cell of a cell, a cell is suspended in the liquid which added 10% of dimethyl sulfoxide (DMSO) to the culture medium, and it is 1 mL to the Ceram tube. After having poured distributively every, putting into BICELL (Nihon Freezer) and making it freeze in -80 ** overnight, it saved in liquid nitrogen. It is 10 mL after performing defrosting in 37-degree C warm water and melting a culture medium. Cell suspension is moved to the centrifuging tube into which the culture medium was put, and it is 10 at 80 xg. After carrying out part at-long-intervals alignment separation, suction removal of the supernatant was carried out with the pipet, and the cell was inoculated into new T-flask.
[0017] 1 .3.4. . Which Measured Cell Density Measurement Approach (Measurement by Dye Exclusion Assay) 19 Viable Cell, and Total Cell Concentration Using Dye Exclusion Assay Which Used Trypan Blue -- this -- 0.2% W/v) of Trypan Blue Water Solutions, and NaCl 4.25% (W/v) of Water Solutions 4:1 (one drop (about 15 mu L) (it carries)) It mixes at a rate, equivalent mixing of the cell suspension is carried out at this liquid, and it is Burker-Turk. Mold counting chamber (ERMA 4296) In . counting chamber which is the approach of carrying out a speculum immediately and measuring viable cell concentration and total cell concentration, the cell concerning two sides, right-hand side and the bottom, was excluded at the time of measurement. 20 which amended the depth of a counting chamber according to the assay value of counting chamber attachment, calculated the volume on a counting chamber, and computed cell concentration here.
[0018] 2 .HPLC Used Drug Metabolism Activity (P450 3 A4 Activity) System-of-Measurement 2.1.HPLC The Used Density Measurement Approach 21 of Substrate and Metabolite - 24 Drug-Metabolism Activity (P450 3 A4 Activity) P450 3 A4 The hormone matter testosterone (the first chemicals; UC-339, molecular weight ; 288.4) metabolized specifically is made into a substrate. 6 which is metabolite Beta-hydroxytestosterone (the first chemicals ;;UC-282, molecular weight ; 304.4) HPLC It evaluated by measuring. HPLC Internal standardization was used as the quantum approach at the time of carrying out. androstenedione (4-Androstene-3, 17-dione) (first chemicals ;;UC-300) was used as internal standard matter here. As introduction standard concentration, they are a substrate, metabolite, and each internal standard matter 100microM. It prepares so that it may become, and it is HPLC. The concentration of metabolite was computed in the substrate list after that based on the peak area of a chart.
0019] - HPLC Conditions HPLC Equipment -- The Shimadzu liquid chromatograph system ; LC10AD;

Column used -- C 18 Inertsil ODS-3V which are a column; (4.6 x150 mm, 5 mu m, GL Sciences Inc.)
** ** buffer -- A Liquid methanol: Distilled water =45 : 55 B Liquid methanol: Distilled water =90 : 10.
Buffer It sets to production and is HPLC. After mixing the ** methanol (Nakarai Tesuku) and distilled water by each ratio, degassing was performed suitably.
** ** -- 1.0 ml/min;
Column temperature -- Room temperature;
** Constant wavelength -- 254 nm;
P450 3 A4 HPLC gradient in activity Conditions [0020]
[Table 1]

時間(分)	A液	B液
0	100%	0%
↓	↓	↓
10	0%	100%
~12	0%	100%
12~	100%	0%

[0021] 2 Quantum P450 3 A4 of the amount of .2. cell total protein Generally activity is pmol 6. Beta-hydroxytestosterone formed/min/mgprotein It is expressed in the unit to say. In case drug metabolism activity is searched for using cell culture supernatant liquid, the amount of cell total protein (mg protein) must be measured. Here, we decided to measure the amount of cell total protein using BCA Protein Assay Reagent Kit (Pierce;23225). The quantum approach followed the attached manual. The adjustment procedure of the cell crushing liquid (crude enzyme liquid) in the amount measurement of cell total protein is shown below. A procedure is shown below.
1) a cell -- the culture medium (in the case of 100 mm dish culture, culture-medium 5 ml is a standard) of a known amount -- suspending -- 500 of them mul a centrifugal tube -- moving -- 1000 xg 10 a part -- between -- centrifugal separation was carried out by 4 **.
2) supernatant liquid -- removing -- 100 mM potassium phosphate buffer (pH7.4) -- suspending -- 1000xg -- 10 a part -- between -- centrifugal separation was carried out by 4 **.
3) By the ultrasonic fungus body destructor (marine electrical machinery T -- A-4200), it is 5. After the sonication during a second, and 1 It ice-cools between parts and is this 4 **********.
4) 14000 xg 10 The quantum of the amount of cell total protein was carried out after centrifugal separation between parts using supernatant liquid by 4 **.
[0022] 2 Pretreatment 25HPLC of .3. cell culture supernatant liquid (sample) It is in charge of using and measuring the substrate and metabolite concentration in cell culture supernatant liquid, and is column Sep-Pak Plus C 18 Waters) for sample pretreatment. It used and the sample was refined. A procedure is shown below. It pours into a column. (apply) The rate of flow at the time of carrying out is 2 - 4 ml/min. It took care so that it might become.
1) -- 100% methanol 2 ml a column -- 2 Time apply It carried out.
2) -- distilled water 2 ml a column -- 2 Time apply It carried out.
3) -- a sample -- 2 ml and apply It carried out.
4) -- distilled water 2 ml a column -- apply It carried out and the column was washed.
5) -- 100% methanol 2 ml apply It carried out and the quality of the specified substance was eluted.
[0023] 3 P450 3 A4 in . animal cell (intact cell) The activity measurement approach 21 and 23110 mL a culture medium RDF (Gln+ or Gln-) -- using -- 100 mm dish a cell -- 1 x10 7 an individual -- seeding was carried out. Culture is the basis of 5 %CO 2 and 37 **, and 16. It carried out time.
2) Concentration is testosterone (it melted to the methanol 100% so that it might be set to 100mM) which culture-medium exchange is performed (amount of culture media 10 mL), and is a substrate 100 mu M It added so that it might become.
3) It is 2 at 5 %CO 2 and 37 **. This culture supernatant 2 mL after carrying out time amount culture It used and activity was measured.
4) After culture termination, the cell was removed by trypsinization and the amount of cell total protein was measured.
[0024]
Example 2] 4 . immanency P450 3 A4 Homo sapiens liver drug-metabolizing enzyme induction medicine rifampicin or the improvement in activity It hits applying an addition culture animal cell to a biotechnology artificial liver, and

is a wild strain HepG2. A cell and gene recombination ammonia metabolic turnover HepG2 built from this invention person's until now P450 3 A4 of the internality in a cell (GS-HepG2 a cell and MSX300 muM resistant strain) Improvement in activity was tried. until now -- HepG2 a cell -- setting -- dexamethasone, phenobarbital, 3-methylcholanthrene, prednisolone, carbamazepine, and rifampicin etc. -- 26, 27, and 28 to which it is reported that capacity to metabolize drugs rises by drug induction if drugs are added to a culture medium. Then, in these drug inducer, it is CYP3A. rifampicin made the most effective as an inducer By adding, it is internality P4503 A4. Improvement in activity was aimed at.

1) 100 mm dish RDF Finally it is 10 mL about a culture medium (FBS is included 10%). It added so that it might become.

2) Wild strain HepG2 It is transgenics GS-HepG2 to a list. About a cell (HNAA-300A cell strain), it is 1 x10 7cells. Seeding was carried out.

3) 37 ** and 5%CO 2 Basis 12 It cultivated time.

4) Culture-medium exchange. 10%FBS Or it contains, it is RDF of a non-blood serum. It is 10 mL about a culture medium. It added.

5) 37 ** and basis 24 of 5%CO 2 It cultivated time.

6) Culture-medium exchange. rifampicin melted to dimethylsulfoxide (DMSO) The last concentration is 10 and 50,100,200,300,500. It is RDF of a non-blood serum so that it may be set to mu M. It adds to a culture medium. It s the non-blood serum RDF as control. It is DMSO to a culture medium. The chisel (0.1%) was added.

7) 37 ** and basis 24 of 5% CO2 After carrying out time amount culture, culture-medium exchange and rifampicin of each concentration were added.

8) Perform 7 for six days (6 culture-medium exchange was performed every day during a day), and it is 200 after that. mu M testosterone It adds to a culture medium and is P450 3 A4. Activity was measured.

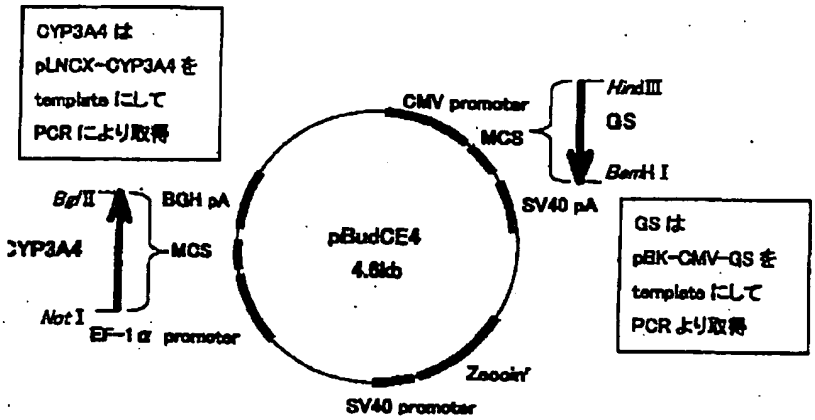
[0025] The above-mentioned wild strain HepG2 Homo sapiens liver drug-metabolizing enzyme induction medicine rifampicin to a cell The result obtained in the addition culture experiment was shown in drawing 1 . This is FBS in a culture medium. rifampicin when it does not contain with the case where it contains Wild strain HepG2 in each concentration P450 3 A4 of a cell Activity is shown. Wild strain HepG2 P450 3 A4 of a cell Activity is 0.6 pmol/min/mg-protein in the condition of not applying drug induction. The value to say was shown. Moreover, it is P450 3 A4 most. It is FBS that whose activity was high. It sets to the included culture medium and is rifampicin 100. muM It is that to which induction was applied and an activity value is 2.3 pmol/min/mg-protein. It became. It s FBS in a culture medium. By comparing the case where it does not contain with the case where it contains, it is FBS. It sets to the culture to include and is internality P450 3 A4. It turned out that it is easy to be guided. FBS important various factors including a growth factor are contained in inside (blood serum). Therefore, FBS By nothing culture, since the important factor for surviving was drained, I thought that it was because the response to change of the external world in a cell becomes blunt.

[0026] Furthermore, gene recombination ammonia metabolic turnover HepG2 Homo sapiens liver drug-metabolizing enzyme induction medicine rifampicin received a cell (GS-HepG2 a cell, MSX300 muM resistant strain) The result obtained in the addition culture experiment was shown in drawing 2 . This is each [when it does not contain with the case where a dialysis blood serum is included] rifampicin. HNAA-300A in concentration P450 3 A4 of a cell strain It is activity. In the condition of not applying drug induction as shown in this drawing, it is HNAA-300A. The cell strain had the almost same activity as wild strain HepG2 cell. However, rifampicin By the drug induction by addition, it is a wild strain HepG2. It compares with a cell and is P450 3 A4. It was checked that induction is hard to be carried out. HNAA-300A A cell strain is a wild strain HepG2. It is pBK-CMV-GS to a cell. MSX after introducing a vector And G418 It uses and selection is applied. It is HNAA-300A if it thinks including the ability to survive also under such a harsh environment. Since the susceptibility over the external world of a cell strain is blunt, it is a wild strain HepG2. It compares with a cell and is rifampicin. P450 3 A4 to depend I thought that it was what induction cannot take place to easily.

[0027]

[Example 3] 5 .P450 3 A4 Construction 5.1.P450 3 A4 of an expression vector Expression vector (pBudCE-GS-CYP3 A4) As an outline 5.1.1. use plasmid use plasmid of construction, it is pBudCE4 29 (Invitrogen;V532-20). It used. This is multicloning site (MCS) 2 KA possession is carried out and it is each MCS. It is human cytomegalovirus immediate-early promoter (CMV) to the upstream. And human elongation 1 Alpha-subsuit (EF-1 alpha) promoter It has. As a marker gene, it is Zeocin at Escherichia coli and an animal cell. It is selectable. P450 3 A4 The outline of construction of an expression vector is shown in the following table 2.

[0028]
[Table 2]



[0029] 5 .1.2. Preparation 5.1.2.1. Use Strain Escherichia Coli TOP10 [(Invitrogen;C 615-00)0030] of Use Plasmid 5 .1.2.2. Culture Medium (LB-Zeocin)
Deionized water 100 mL Bacto-tryptone 1 (Difco) g, Bacto yeast extract0.5 (Difco) g, and NaCl 0.5 g pH 7.0 after melting The autoclave was prepared and carried out. It is 2 g when making it a plate. Agar It added. It is Zeocin if it becomes at least 55 ** after an autoclave. 50 mug/mL It added so that it might become.
[0031] 5 the creation 1 of the transformation and competent cel of the Escherichia coli by the .1.2.3. calcium chloride method -- the Escherichia coli which carries out a transformation -- 5 mL LB Shaking culture was carried out by 37 ** by the culture medium overnight.
2) -- preculture liquid 2 mL 40 mL LB a culture medium -- inoculation -- carrying out -- 37 **2 Time amount culture was carried out.
3) -- the inside of ice -- ten a part -- the above -- leaving it -- 4 **, 6000 xg, and 5 The harvest was carried out by carrying out part at-long-intervals alignment separation.
4) -- 50 mM CaCl 2 20 mL which ice-cooled precipitation suspending -- 0 **20 It was left between parts.
5) -- centrifugal separation -- after a harvest and 50 mM CaCl 2 4 mL suspending -- 200 muL It poured distributively every.
- Transformation 200 muL It is DNA to a competent cel. A solution is added a suitable amount (0.01 mug extent), and it is 1 in ice. Time amount neglect was carried out. 42 At **, it is 90. After giving the heat shock during a second, it quenches by iced water, and it is 0.8 mL. LB A culture medium is added and it is 1 at 37 **. Time amount shaking culture was carried out. 100 among this culture medium muL LB-Zeocin It extended to the culture medium and cultivated by 37 ** overnight.
[0032] 5 Plasmid DNA from .1.2.4. Escherichia Coli Preparation (Alkali Extraction Method)
5 mL LB-Ampicilin Inoculation of the Escherichia coli transformant is carried out to a liquid medium, and it is 16 at 37 **. Time amount shaking culture was carried out. It is 1.5 mL.about culture medium. Extent picking, 12000 xg, and 2 Part at-long-intervals alignment separation was carried out, and the harvest was carried out. solution I100 after stirring precipitation with a vortex mixer It suspends completely in muL and is solution II200. muL It is 3-4 quietly moreover. Time fall mixing is carried out and it is 5 correctly in ice. It was left between parts. It is solution II cooled beforehand 150 muL In addition, it mixes violently and is 5 in ice. It was left between parts. 12000 xg and 5 After carrying out part at-long-intervals alignment separation, supernatant liquid was moved to a new sample tube. 20 after performing a phenol-chloroform extraction and performing ethanol precipitation further about this supernatant liquid mug/mL Dnase free RNase TE 50 included muL It dissolved.
solution I: 50 mM Glucose, 25 mM Tris--Cl (pH 8.0), 10 mM EDTAsolution II: 0.2 N NaOH and 1%%SDS (it prepares at the time of an important point)
solution III: 5 M Acetic-acid water solution containing potassium acetate [0033] 5 .2. gene relation -- fundamental -- actuation 5.2.1. agarose electrophoresis 1/2 xTAE It carried out by 0.8% agarose gel (TaKaRa;Agarose LO3) using the buffer solution. The small electrophoresis tub (Advance Co.Ltd.;Mupid2) was used as a migration tub. A sample is usually DNA. It is 1/10 to a solution. xten of an amount The stain solution for migration was added and produced. Migration is constant-voltage 100 V. 40 The part was performed. They are after migration and gel with an ethidium bromide water solution (EtBr) (0.5 mu g/ml) 10 It dyes between parts and is DNA at Tran Swi Rumi Noether (Ultra Violet C62). The band was observed. A photograph is UV. A photograph was taken with the Polaroid

(trademark) camera using the filter and the red filter.

[0034] 5 .2.2. ethanol precipitate DNA To the included solution, it is 1/10. 3 M of an amount A sodium acetate solution (pH 5.2) is added and it is 2.5 of the solution further. The 100 % ethanol of the amount of double is added, and it mixes, and is 20 at a room temperature. It was part-left. It is this 18000 xg and 10 Part at-long-intervals alignment separation was carried out, and supernatant liquid was removed. It is 70% ethanol to this precipitate Optimum dose ****, 18000 xg, and 10 After carrying out part at-long-intervals alignment separation, supernatant liquid is removed, and they are the sterilized water of after reduced pressure hardening by drying and optimum dose, or Sterilization TE about precipitate. It dissolved in the buffer solution (10 mM Tris-Cl (pH 7.5) and 1 mMEDTA).

[0035] 5 .2.3. phenol chloroform-extraction DNA It is TE about a solution. It was saturated with the buffer solution and phenol chloroform isoamyl alcohol (25:24:1) mixed liquor was mixed equivalence, in addition well. this -- 18000 xg -- at-long-intervals alignment separation was carried out for 10 minutes, and the upper layer was moved to a new sample tube.

[0036] 5 .3.PCR P450 3 A4 to depend A gene and GS Preparation / use device GeneAmpR PCR System 2400 and (PERKIN ELMER) templateP450 3 A4 of a gene About a gene, they are pLNCX-CYP3 A4 (Els M.De Grone it presents from a teacher), and GS. About a gene, it is PBK-CMV-GS. It used. Used primer is shown in the following table 3.

[0037]

[Table 3]

GS遺伝子をPCRにより獲得する際に用いたプライマー

N末端側 *Hind*III

5' - AAAAAA**AAGCTT**ACCATGGCCACCTCAGCAAGTTCCC - 3'

C末端側 *Bam*H I

5' - CCCCCC**GGATCC**AATTAGTTTTTGTATTGGAAGGGCTC - 3'

CYP3A4をPCRにより獲得する際に用いたプライマー

4末端側 *Not* I

5' - TTTTTT**GCGGCCGC**GTGATGGCTCTCATCCCAGACTTGG - 3'

C末端側 *Bgl*II

5' - GGGGGG**AGATCT**ATTCAGGCTCCACTTACGGTGCCATC - 3'

[0038] In addition, it is Kozak so that it may be in charge of the design of a primer and the translation in an animal cell may be started more correctly. It referred to the array (A/G NN ATG G). Moreover, it is each PCR to the following table 4. A presentation and conditions of a reaction were shown.

[0039]

[Table 4]

primer GS *Hind*III	(20 pmol/μl)	1 μl	primer CYP3A4 *Not* I	(20 pmol/μl)	1 μl
primer GS *Bam*H I	(20 pmol/μl)	1 μl	primer CYP3A4 *Bgl*II	(20 pmol/μl)	1 μl
Template (pBK-CMV-GS)	(50 pmol/μl)	1 μl	Template (pLNCX-CYP3A4)	(50 pmol/μl)	1 μl
KOD polymerase buffer (×10)		5 μl	KOD polymerase buffer (×10)		5 μl
dNTP 2 mM		5 μl	dNTP 2 mM		5 μl
$MgCl_2$ 25 mM		2 μl	$MgCl_2$ 25 mM		2 μl
KOD polymerase 2.5 unit/μl		1 μl	KOD polymerase 2.5 unit/μl		1 μl
滅菌水		34 μl	滅菌水		34 μl
total		50 μl	total		50 μl

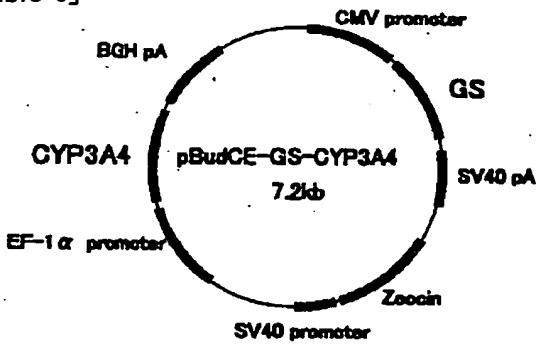
PCR条件

98℃ 15 sec → 65℃ 2 sec → 74℃ 30 sec

← 25 cycles →

[0040] 5 .4.pBudCE4 P450 3 A4 which is a vector (invitrogen) and an insertion A gene and GS pBudCE4 prepared by ligation5.1.2.4. with a gene The P450 3 A4 gene and GS which were prepared by the vector and 5.3. It decided to carry out ligation using a gene. This process is TAKARA SHUZO CO., LTD. An order was placed with the gene-analysis pin center,large. Consequently, obtained P450 3 A4 Expression vector (pBudCE-GS-CYP3 A4) It is shown in the following table 5. This vector is pBudCE4. To two multicloning site of a vector, it is pLNCX-CYP3 A4. CYP3 A4 (gene of P450 3 A4) and pBK-CMV-GS of the origin GS of the origin A gene is inserted. .

[0041]
[Table 5]



[0042] Furthermore, 5.3. is followed and it is P450 3 A4. A gene and GS It is PCR about a gene. It amplifies and is PCR. Product 4 muL Electrophoresis was carried out using 0.8 % agarose gel. The result is shown in drawing 2.. Rain ** is P450 3 A4. Gene (about 1.5 kbp(s)) and rain ** is GS. Gene (about 1.1 kbp(s)) and rain ** is a ambda/Hind III marker. P450 3 A4 which is the target gene from this A gene and GS Acquisition of a gene was checked. P450 3 A4 P450 3 A4 inserted in the expression vector The sequence result of a gene (CYP 3 A4) is shown in the following table 6. Di CYP3 A4 registered into - TABESU Base sequence 33 When it compares, it is 6 after an initiation codon (ATG). The base eye was not C (cytosine) but A (adenine). However, from configuration amino acid serving as a leucine with both (CTC and CTA), in case it is made to translate into protein, it is thought that P450 3 A4 inserted in a vector has original activity.

[0043]
[Table 6]

N末端側)TCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCA *Not I* 開始コドン
AGTTTTTTTCTTCCATTTCAGGTGTCGTGAACACGTGGTC*GCGGCCGC*GTGATGGCTCT
ATCCCAGACTTGGCGATGGAAACCTGGCTTCTCCTGGCTGTCAGCCTGGTGCTCCTCTA
CTATATGGAACCCATTCACATGGACTTTTTAAGAAGCTTGGAATTCCAGGGCCCACACC
CTGCCTTTTTTGGGAAATATTTTGTCCTACCATAAGGGCTTTTGTATGTTTGACATGGA
TGTCATAAAAAGTATGGAAAAGTGTGGGGCTTTTATGATGGTCAACAGCCTGTGCTGGC
ATCACAGATCCTGACATGATCAAAACAGTGCTAGTGAAAGAATGTTATTCTGTGTTCAG
AACCGGAGGCCTTTTGGTCCAGTGGGATTTATGAAAAGTGCCATCTCTATAGCTGAGGA
GAAGAATGGAAGAGATTACGATCATTGCTGTCTCCAAGCTTCAACAGTGGAAAACTCAA
GAGATGGTCCCTATCATTGCCCAGTATGGAGATGTGTTGGTGAGAAATCTGAGGCGGGA
GCAGAGACAGGCAAGCCTGTCACCTTGAAAGACGTCTTTGGGGCCTACAGCATGGATGT
ATCACTAGCACATCATTTGGAGTGAACATCGACTCTCTCAACAATCCACAAGACCCCTT
GTGGAAAACACCAAGAAGCTTTTAAGATTTGATTTTTTGGATCCATTCTTTCTCTCAAT
ACAGTCTTTCCATTCCTCATCCCAATTCTTGAAGTATTAAATATCTGTGTGTTTCCAAG
GAAGTTACAAATTTTTTAAGAAAATCTGTAAAAAGGATGAAAGAAAGTCGCCTCGAAGA
ACACAAAAGCACCGAGTGGATTTCCTTCAGCTGATGATTGACTCTCAGAATTCAAAAGA
ACTGAGTCCCACAAAGCTCTGTCCGATCTGGAGCTCGTGGCCCAATCAATTATCTTTAT
TTTGCTGGCTATGAAACCACGAGCAGTGTTCTCTCCTTCATTATGTATGAACTGGCCAC
CACCCTGATGTCCAGCAGAAACTGCAGGAGGAAATTGATGCAGTTTTACCCAATAAGGC
CCACCCACCTATGATACTGTGCTACAGATGGAGTATCTTGACATGGTGGTGAATGAAAC
CTCAGATTATTCCCAATTGCTATGAGAGTTGAGAGGGTCTGCAAAAAAGATGTTGAGAT
AATGGGATGTTCATTCCCAAAGGGTGGGTGGTGATGATTCCAAGCTATGCTCTTCACCG
GACCCAAAGTAGTGGACAGAGCCTGAGAAGTTCCTCCGTGAAAGATTCAGCAAGAAGAA
AAGGACAAGATAGATCCTTACATATACACACCCTTTGGAAGTGGACCCAGAAACTGCAT
GGCATGAGGTTTGCTCTCATGAACATGAAACTTGCTCTAATCAGAGTCCTTCAGAAGTT
TCCTTCAAACCTTGTAAAGAAACACAGATCCCCGTGAAATTAAGCTTAGGAGGACTTCT
CAACCAGAAAAACCCGTTGTTCTAAAGGTTGAGTCAAGGGATGGCACCGTAAGTGGAGC
停止コドン
BglII
TGAAT*AGATCT*GGCCGGCTGGGCCCGTTTCGAAGGTAAGCCTATCCCTAACCCTCTCCT
CGGTCTCGATTCTACGCGTACC

[0044] Moreover, P450 3 A4 GS inserted in the expression vector The sequence result of a gene is shown in the following table 7. GS About a gene, it is template. pBK-CMV-GS carried out GS Array 30 of a gene It was

completely the same. pBK-CMV-GS Introduced CHO A cell (CN9-500-4 cell strain) and HepG2 It sets into a cell (HNAA-300A cell strain), and is GS. GS inserted in this vector from activity being checked It is thought that it has the function as original protein.

[0045]

[Table 7]

N末端側)TCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATA

GGGAGACCC*AAGCTT*ACC*ATG*GCCACCTCAGCAAGTTCCCACGTTGAACAAAAACATCAAG

*Hind*Ⅲ 開始コドン

CAAATGTACCTGTGCCTGCCCCAGGGTGAGAAAGTCCAAGCCATGTATATCTGGGTTGAT

GGTACTGGAGAAGGACTGCGCTGCAAAACCCGCACCCTGGACTGTGAGCCCAAGTGTGTA

GAAGAGTTACCTGAGTGGAATTTTGATGGCTCTAGTAGCTTTCAGTCTGAGGGCTCCAAC

AGTGACATGTATCTCAGCCCTGTTGCCATGTTTCGGGACCCCTTCCGCAGAGATCCCAAC

AAGCTGGTGTTCTGTGAAGTTTTCAAGTACAACCGGAAGCCTGCAGAGACCAATTTAAGG

CACTCGTGTAAACGGATAATGGACATGGTGAGCAACCAGCACCCCTGGTTTGGAATGGAA

CAGGAGTATACTCTGATGGGAACAGATGGGCACCCTTTTGGTTGGCCTTCCAATGGCTTT

CCTGGGCCCCAAGGTCCGTATTACTGTGGTGTGGGGCCAGACAAAGCCTATGGCAGGGAT

ATGGTGGAGGCTCACTACCGCGCCTGCTTGTATGCTGGGGTCAAGATTACAGGAACAAAT

GCTGAGGTCATGCCTGCCCAGTGGGAATTCCAAATAGGACCGTGTGAAGGAATCCGCATG

GGAGATCATCTCTGGGTGGCCCGTTTCATCTTGCATCGAGTATGTGAAGACTTTGGGGTA

ATAGCAACCTTTGACCCCAAGCCCATTCCTGGGAACTGGAATGGTGCAGGCTGCCATACC

AACTTTAGCAACCAAGGCCATGCGGGAGGAGAATGGTGTGAAGGACATCGAGGAGGCCATC

GAGAAACTAAGCAAGCGGCACCGGTACCACATTCGAGCCTACGATCCGAAGGGGGGCCTG

GACAATGCCCGTCGTCTGAGTGGGTTCCACGAAACGTCCAACATCAACGACTTTTCTGCT

GGTGTCGCCAATCCGCAGTCCAGCATCCGGATTCCCCGGACTGTCGGCCAGGAGAAGAAA

GGTTACTTTGAAGACCGCCGCCCCTCTGCCAATTGTGACCCGTTTGCAGTGACAGAAGCC

ATCGTCCGCACATGCCTTCTCAATGAGACTGGCGACGAGCCGTTCCAATACAAAAACTAA

TT*GGATCC*GAACAAAAACTCATCTCAGAAGAGGATCTGAATATGCA (C末端側) 終止コドン

*Bam*H I

[0046]

[Example 4] 6 .P450 3 A4 Introductory P450 3 A4 to the animal cell of an expression vector It is a wild strain HepG2 about an expression vector. Cell (ATCC No.HB-8065) And gene recombination ammonia metabolic turnover HepG2 already built by this invention person It tried to introduce into a cell (HNAA-300 A share). It is P450 3 A4 to an animal cell. In introducing an expression vector, this vector must be prepared by the high grade. Then, Wizard PureFection Plasmid DNA Purification System (A2160; how to make DNA stick to a magnet and to collect plasmid DNA s of a high grade after removing endotoxin etc.) was used. The approach followed the attached manual (the publication of an approach is omitted). P450 3 A4 of the high grade obtained above An expression vector is used and it is transfection. It carried out. transfection The approach used the liposome method (TaKaRa and Trans IT Polyamine Transfection Reagents).

1) It is 35 mm dish about a 4 x10 5 piece cell. It cultivated by seeding and 37 ** overnight. As a culture medium, they are HepG2 and CHO-K1. It sets and is FBS. About an entering RDF (Gln+) culture medium, they are HNAA-300A and CN 9-500-4. It set and the RDF (Gln-) culture medium containing a dialysis blood serum was used.

2) The next day and 100 mu l It is TransIT Transfection Reagent to a serum free medium. 20 mu l It is vortex moreover. It mixed.

3) It is 10 min at a room temperature, It cultivated.

4) It is vector DNA 3 to this. mu g It is pipetting quietly moreover. It carried out.

5) It is 10 min at a room temperature, It cultivated.

6) What was prepared by 5 was added with POTAPOTA to the cell culture medium prepared by 1. dish It shook slowly and was made to mix.

7) 72 It cultivated by time amount and 37 **.

8) Carry out trypsinization of what was prepared by 7, remove it, and it is smallness about the whole quantity. - T Seeding was carried out to the flask (area-of-base 25cm 2). A culture medium is Zeocin. The passage was carried out using the RDF (Gln+ and Gln-) culture medium which is not included.

9) Smallness - T Zeocin after becoming confluent in a flask It planted in the included selective medium and inherited. Zeocin in a selective medium concentration -- HepG2 And HNAA-300A **** -- 200 mug/ml ** -- it carried out.

[0047] In this way, obtained P4503 A4 HepG2 made to discover Cell (CYP3 A4-GS-HepG2) P450 3 A4 The result of having measured activity is shown in Table 8. in addition, in front Naka, "P450 3 A4-HepG2" is written (the

nside of each table of a following and book specification -- the same). [this cell]

0048]

Table 8]

細胞株	P450 3A4 活性 (testosterone 6β-hydroxylation 活性 pmol/min/mg-protein)
P450 3A4-HepG2	490
HepG2(薬物誘導なし)	0.6
HepG2(薬物誘導あり)	2.3

0049] clear from Table 8 -- as -- P450 3 A4 Installation HepG2 the activity value in a cell -- about 800 of a wild strain twice -- a high value and 490 pmol/min/mg-protein It was shown. P450 3 A4 CHO made to discover even if t compares with the activity in a cell (P450 3 A4-CHO cell strain) -- P4503 A4-HepG2 the activity in a cell strain -- about 20 twice -- it means that the high value was shown P450 3 A4 Installation CHO It compares with a cell and is P450 3 A4. Installation HepG2 P450 3 A4 in a cell Activity is 20. Although it became high more than twice This is an environment (for example, it sets for oxidation / reduction reaction by P450) which supports the reaction mechanism of CHO. It compares with a cell and is HepG2. It sets into a cell and is P450. P450 P450 from a reductase It is thought that it is because it remains [that electronic supply is performed actively etc. and] in ntracellular.

0050] It is 24 here. P450 3 A4 of the first hepatocyte which carried out time amount culture An activity value is 252.8 pmol/min/mg-protein. 35 reported. Moreover, at the thing using rat founder hepatocyte, it is P450 3 A4 after 4-hour culture. Activity is 407 pmol/min/mg-protein and 24. The activity after time amount culture is 158 pmol/min/mg-protein. 36 which has become. It is P450 3 A4 from this. Installation HepG2 It can be said that the cell strain had the first hepatocyte, an EQC, or the activity beyond it. However, in vivo P450 3 A4 of the Homo sapiens hepatocyte which can be set For activity, individual difference is 1000 of a certain thing - 1500 pmol/min/mg-protein. It is called extent, turns to 37 and the clinical application to Homo sapiens, and is P450 3 A4-HepG2 further. P450 3 A4 of a cell I thought that he wanted to raise activity further.

0051]

Example 5] 7 .P450 3 A4 GS in a manifestation animal cell Gene amplification approach P450 3 A4 using a genetic system P450 3 A4 in a manifestation animal cell It is GS in order to raise activity. Gene amplification in a genetic system was performed.

1) -- Smallness-T a flask (area-of-base 25 cm 2) -- setting -- HepG2 a cell -- 200 mug/mlZeocin And MSX of each concentration the included selective medium -- a cell -- about -- 5 x10 five-piece seeding was carried out, and culture-medium exchange was performed until it became confluent.

2) -- the selective medium which the cell was exfoliated [selective medium] when becoming confluent, and raised MSX concentration (Methionine Sulfoximine) -- about 5x105 The stroke of carrying out individual seeding was repeated and the gene amplification in a hetero condition was tried.

0052] Consequently, P450 3 A4 Installation HepG2 It sets into a cell and is each MSX. The resistant strain of concentration was acquired. Next, it is P450 3 A4 about each resistant strain. Activity was measured (3). The result is shown in drawing 3 . In addition, MSX P450 3 A4 Since activity may have been affected, in the passage before activity measurement, MSX was not added to a culture medium. Moreover, it is MSX also to the culture medium at the time of carrying out activity measurement. It did not add. MSX It is GS by addition. P450 3 A4 using a gene amplification system Although improvement in activity was aimed at, the almost same activity value was shown also in which resistant strain. From this, it is GS. The gene amplification in a gene is used and it is P450 3 A4. It can be said that activity was not able to be raised. Each cell strain is MSX why here. In spite of having acquired resistance, it considered as follows that P450 3 A4 activity did not rise.

1) P450 3 A4 GS introduced into the expression vector Since it is not by the gene and gene amplification happened in GS gene of internality, it is P450 3 A4. Activity did not rise (P450 3 A4 activity is change nothing and GS in this case activity will rise).

2) P450 3 A4 GS introduced into the expression vector Although gene amplification happened in the gene, it is P450 3 A4 at a certain factor. A gene did not amplify (P450 3 A4 activity is change nothing and GS in this case activity rises).

3) GS The gene amplification in a genetic system itself had not worked (it is P450 3 A4 in this case activity or GS activity does not change, either). That is, by devices other than gene amplification (for example, film variation etc.), t is MSX. It is thought that resistance was acquired.

0053]

Example 6] 8 .P450 3 A4 Installation HepG2 GS in a cell the activity measurement method 32 -- in order to verify these things -- each MSX Concentration resistance P450 3 A4 Installation HepG2 GS of a cell It decided to evaluate activity.

3 .1.GS Activity measurement principle GS Gamma-glutamyl The catalyst of the transition reaction is carried out. namely, hydroxylamine from -- gamma-glutamylhydroxyamate generating -- this gamma-glutamylhydroxyamate ferric chloride Addition shows characteristic brown. This is hydroxylamine. Used glutamine synthetase It is the principle of activity measurement. This reaction is used and it is GS. Activity was measured.

0054] 8 .2.GS He is TBS about activity measurement approach 1 cell suspension. 500 once washing muL midazole buffer It suspended.

2) 10% beta-mercaptoethanol (.) 5 muL It added. (antioxidant)

3) an ultrasonic fungus body destructor (T-A-4200; marine electrical machinery incorporated company) -- 10 After the sonication during a second, and 1 a part -- between -- ice-cooling -- 3 ********** -- the cell was crushed by things.

4) 10% beta-mercaptoethanol It is 5 again. muL It added.

5) It is 100 mM phenylmethylsulfonylfluoride (PMSF; ethanol solution) 5 muL It added.

6) It is 200 mM Pepstatin A (ethanol solution) 5 muL It added. (PMSF and Pepstatin A are protease inhibitors)

7) 18,000xg.5 Centrifugal separation was carried out by 4 ** between parts.

8) Supernatant liquid was moved to new EPPEN and the volume was measured.

9) It is 15 in 37 ** correctly [after adding and carrying out the vortex of the reaction substrate liquid to crude enzyme liquid]. It was made to react between parts.

10) 0.75 mL FeCl3 solution is added and it is 5 at 18,000 xg. After carrying out part at-long-intervals alignment separation, supernatant liquid is struck to a cuvette, and it is A 535. The absorbance was measured. 11) Measured value to blank The value was lengthened and activity was searched for. the bottom of this Measuring condition -- A 535 =0.340 the time -- 1 unit ** -- 32 carried out. It is gamma-glutamyl transfer to Table 9. About the presentation of an activity measurement reagent, it is FeCl 3 in Table 10. The presentation of a solution was shown.

0055]

Table 9]

	Stock液の組成	測定時における添加量
Imidazole-HCl	100 mM, pH7.2	250μL
$MnCl_2$	125 mM	20 μL
L-Glutamine	250 mM	100 μL
arsenate	200 mM, pH7.2	50 μL
Hydroxylamine	1 M, pH7.2	50 μL
ADP・Na	2.5 mM	20 μL
酵素液		10 μL
Total volume		500 μL

0056]

Table 10]

	Stock液の濃度	測定時における添加量
$FeCl_3$	1.11 M	250 μL
HCl	2.01 M	250 μL
Trichloroacetic acid	0.60 M	250 μL
Total volume		750 μL

0057] Moreover, GS Since it is the purpose to measure activity, it is GS. Extinction values other than an enzyme reaction should be removed. Then, blank as shown in Table 11 It took.

0058]

Table 11]

	b_1	b_2	b_3
Imidazole-HCl	O	O	O
$MnCl_2$	O	O	O
L-Glutamine	×	O	×
arcenate	O	O	O
Hydroxylamine	×	O	×
ADP·Na	×	O	×
酵素液	O	×	×

0059] Notes in addition b 1, b 2, and b 3 blank A class is shown. Moreover, it is shown that O of the above-nentioned table does not add addition and x. b 1 The thing and b 2 which extracted the substrate The thing and b 3 which extracted the enzyme A substrate and an enzyme are extracted. Volume adds ultrapure water and is 500. nuL It carried out. blank at the time of enzyme activity measurement finally It is the following, and made and calculated.

blank =(b 1)+(b 2)-(b 3) [0060] P450 3 A4 in the culture which used the synthetic medium for drawing 4 Installation HepG2 GS of a cell (each MSX concentration resistant strain) The result of activity (inside of a synthetic medium) s shown. Each MSX Concentration resistance P450 3 A4 Installation HepG2 GS in a cell The difference was not found out by activity. From this, it sets to each MSX concentration resistant strain, and is P450 3 A4. It is GS that there was no difference in activity. It thinks because the gene amplification in a genetic system had not happened well. It is thought that each [these] MSX concentration resistant strain acquired resistance according to devices other than gene amplification (for example, film variation etc.). A gene amplification system will be used from now on, and it is P450 3 A4. Considering raising activity, it is GS. Gene amplification systems other than a gene (CAD genetic system etc.) must be used.

0061]

Example 7] 9 P4503 A4 in . blood serum Installation HepG2 P450 3 A4 in a cell In clinical application, the plasma of Buta or Homo sapiens will touch the activity measurement method 30 with a gene recombination animal cell in KIGUNASU (cell culture equipment) in time. Then, it is P450 3 A4 as a form more near application with the following approaches. Installation HepG2 It decided to evaluate the capacity to metabolize drugs (P450 3 A4 activity) of a cell by culture using not a synthetic medium but the blood serum which is a component more near plasma. Moreover, each MSX About the concentration resistant strain, activity was measured similarly.

) 100 mm dish It is a cell 1 x10 7 An individual, seeding. The culture medium used RDF (Gln+). The amount of culture media is 10 mL. 5 The basis of %CO 2 and 37 **, and 16 Time amount culture was carried out.

) Sample a culture medium and they are 10mL(s) about a blood serum (FBS). testosterone which is a substrate in addition Concentration is 100. mu M It added so that it might become.

) The basis of 5 %CO 2 and 37 **, and 2 Time amount culture. This culture supernatant 2 mL It used and activity was measured.

) After culture termination, the cell was removed by trypsinization and the amount of cell total protein was measured.

0062] The obtained result is shown in drawing 5 . It also sets to which cell strain and is P450 3 A4. Activity is 200 pmol/min/mg-protein. The value of order was shown. This is P450 3 A4 measured in the synthetic medium. Activity is 5 about 2/. It was a value. However, MSX GS by addition P450 3 A4 of genetic system gene amplification The effectiveness in activity was not seen.

0063]

Example 8] 10 .P450 3 A4 Installation HepG2 Measuring method 31 former P450 3 A4 of the lidocaine metabolic turnover ability in a cell As a substrate when measuring activity, testosterone (hydroxylation like 6 beta) which is the hormone matter was used. P450 3 A4 Installation HepG2 It is testosterone when it assumes using a cell as a biotechnology artificial liver. It is necessary to evaluate also about the metabolic turnover of the drug of an except check). Then, it decided to measure the metabolic turnover ability of the lidocaine currently used widely in case biotechnology artificial liver capacity to metabolize drugs is evaluated. Lidocaine is used as the model substrate of the first phase reaction, and a clinical index at the time of liver failure.

· HPLC Conditions HPLC Equipment -- Shimadzu LC10AD Liquid chromatograph system-usage column -- C 18 nertsil ODS-3V which are a column () [4.6 x150 mm, 5 mu m,] [GL Sciences] Inc. ** 20 mM NaClO 4 (pH 2.5) 15%acetonitrile [-- 2.0 ml/min column temperature / -- ****** / Law] style (degassing was performed) ** wavelength -- 205 nm [0064]) ** buffer -- The result is shown in Table 12. this table shows -- as -- P450 3 A4

Installation HepG2 the lidocaine metabolic turnover ability in a cell -- 5.1 nmol/min/mg-protein it was . In addition, a value is the 2 times measurement average and each measured value is 4.7 and 5.5.

[0065]

[Table 12]

細胞株	P450 活性 (lidocaine 3- hydroxylation 活性, nmol/min/mg-protein)
P450 3A4-HepG2	5.1
HepG2	<0.1

[0066]

[Example 9] 11. It examined whether it would continue at the maintenance of drug metabolism activity over a long period of time, and also a long period, and the cell strain of this invention could maintain activity. Therefore, HepG2 incorporating CYP3 A4 and GS which are the cell strain of this invention was cultivated more than for 80 days (about 20 or more generations), and it measured about the drug metabolism activity. The obtained result is shown in the following table 13. Consequently, it became clear that the high activity of about 426 pmol/min was maintained per protein mg.

[0067]

[Table 13]

Table GS-P450 3A4 発現ベクター導入の効果

	P450 3A4 活性 (testosterone 6β-hydroxylation activity) (pmol min^{-1} $mg\text{-}protein^{-1}$)
CHO-K1 細胞(野生株)	< 0.1
P450 3A4 導入 CHO 細胞	21
HepG2 細胞 (野生株)	0.6
P450 3A4 導入 HepG2 細胞	490
P450 3A4 導入 HepG2 細胞(100%血清培養)	200
P450 3A4 導入 HepG2 細胞(80 日間以上、約 40 世代経代後)	426
ヒト初代肝細胞(生体より単離 24 時間後)	250*
ヒト初代肝細胞(生体より単離 96 時間後)	11**
ヒト生体肝	1000～1500***

*Journal of Hepatology 31:540 (1999)
**BiochemicalSocietyTransactions 22:131S (1994))
***Biochemical Pharmacology 40:2525 (1990)

[0068]

[Example 10] 12 evaluation 12.1 of the capacity to metabolize drugs as a biotechnology artificial liver based on . path clearance the path clearance theory of session -- here -- P450 3 A4 Installation HepG2 It decided to evaluate the capacity to metabolize drugs as a biotechnology artificial liver at the time of applying this cell to KIGUNASU (cell culture equipment) based on the lidocaine metabolic turnover ability of a cell. Moreover, 38, 39, and 40 it is in charge of this evaluation, and using path clearance as an index. Generally path clearance shows the magnitude of the capacity for an organ to process a drug. Processing here is a view including all, such as not only a metabolic turnover but migration which penetrates disappearance and the biomembrane of a drug. Path clearance (CL) (ml/min) is the concentration in blood (C) (mg/ml). When a drug is processed at a rate Vel (mg/min), it defines as a degree type.

Vel =CL and C -- here, it is expressed with a bottom type when the path clearance on the basis of drug concentration C in in blood (inside of plasma) (mg/ml) which flows into a biotechnology artificial liver is defined as biotechnology artificial hepatic clearance (CL artificial liver) (ml/min).

Vel =CL An artificial liver and C in [0069] 12 Plasma of Drug Concentration C in (Mg/Ml) Flows into Biotechnology Artificial Liver by the Rate of Flow Q (Ml/min) Here. Count of .2. Path Clearance -- P450 3 A4 Installation HepG2 Considering the model which drug concentration becomes small with C out (mg/ml) by the metabolic turnover by the cell, and flows out The inflow rate V in (mg/min) and exit velocity V out (mg/min) of a drug The processing

speed Vel (mg/min) in a biotechnology artificial liver is an inflow rate of a drug, respectively. : Exit velocity of V in =Q and C in drug : V out =Q and processing speed in a C out biotechnology artificial liver : Vel =CL It is expressed an artificial liver and C in. It is the amount change rate of drugs in a biotechnology artificial liver (V) from this. (mg/min) is V = Vin-Vout-Vel=Q(C in-C out)-CL. An artificial liver and C in .... It is expressed **. At a steady state, since there is nothing, drug concentration change within a biotechnology artificial liver is V = 0 here. It becomes and is CL. Artificial liver = it is set to Q-(C in-C out)/C in.

Here (C in-C out) /C in=Er .... It is CL if it sets with **. Artificial liver = Q-Er .. It becomes **. Er Extraction efficiency, a call, and this express the rate that a drug is processed, while plasma passes a biotechnology artificial liver.

[0070] 12 In case it asks for the related biotechnology artificial hepatic clearance (CL artificial liver) of .3. biotechnology artificial hepatic clearance and intrinsic clearance, intrinsic clearance (CL int) must be considered. The throughput which a biotechnology artificial liver originally owns with intrinsic clearance first on the basis of the effective drug concentration (Ce) of the minute part where the drug in a biotechnology artificial liver disappears -- so to speak -- P450 3 A4 Installation HepG2 What is necessary is just to think that the throughput in a cell is expressed. As an assumption, the uncombined mold drug concentration (f and C) which has not been combined with the plasma protein in plasma etc. has the uncombined mold drug concentration of the processing part in a biotechnology artificial liver, and the relation of a concentration balance, and presupposes that only an uncombined mold drug is processed here. Here, it is f. A binding fraction with the plasma protein of a drug is expressed. if it thinks that the effective drug concentration in a biotechnology artificial liver (Ce) is uniform, and equal to the uncombined-among plasma mold concentration (f-C out) flowing out -- f and C out=Ce ** Vel =dn/dt =CL It becomes an artificial liver, C in =CL int, Ce=CL int, f, and C out ....**.

[0071] 12 Set at a ** ceremony in a .4. biotechnology artificial hepatic clearance steady state, and it is V = 0. They are [becoming and] Q(C in-C out) =CL int, f, and C out from ** type. It becomes.

From this formula and ** type, it is extraction efficiency Er. It becomes Er =f and CL int/(Q+f and CL int), and is CL from ** type. Artificial liver =Q -f and CL int/(Q+f and CL int) .... It becomes **.

[0072] 12.5 . intrinsic clearance P450 3 A4 Installation HepG2 if the drug metabolism by the cell assumes that a Michaelis-Menten equation is followed -- the substrate concentration of a metabolic turnover part, i.e., the effective drug concentration in a biotechnology artificial liver, -- Ce and Ce the maximum metabolic rate of the drug in infinity -- V max and a Michaelis constant -- K m ** -- if it carries out, it will become Vel =V max and Ce/(K m+Ce). Moreover, from being expressed with ** type, intrinsic clearance is CL int =V max/(K m+Ce).... It is expressed **.

[0073] 12 Trial calculation (cell-culture equipment: about [number of cells] 4 x109 cells and volume 1 L) 41 and 42 of .6. biotechnology artificial hepatic clearance, for example, KIGUNASU which is indicated by JP,06-113818,A, P450 3 A4 The trial calculation of biotechnology artificial hepatic clearance was made having assumed that it was filled up with introductory HepG2 cell. Already measured P450 3 A4 K m =65 in lidocaine metabolic turnover ability 5.1 nmol/min/mg-protein in introductory HepG2 cell, and a lidocaine metabolic turnover muM43 (for reference discount) And lidocaine average concentration Ce=25 in the blood in the effective lidocaine concentration in a biotechnology artificial liver, i.e., a steady state, muM43 To a radical, it is CL int from ** type. The value was computed. Consequently, CL int =57 ml/min It was able to be found. this value and flow-velocity Q=15 ml/min 41 in KIGUNASU (cell culture equipment) and -- if the trial calculation of biotechnology artificial hepatic clearance is made based on binding fraction (reference value) f =0.3 43 with the plasma protein of lidocaine -- CL Organ = 8.0 ml/min It was able to be found. This showed the value almost equivalent to the biotechnology artificial liver (CL =7.0 ml/min) 44 which used rat founder hepatocyte. Therefore, P450 3 A4 Installation HepG2 It turned out that the biotechnology artificial liver using a cell has the function of the same order as the biotechnology artificial liver using the first hepatocyte in drug metabolism. The first hepatocyte is P450 3 A4 which has reproductive integrity, when the supply from a living body is required and it takes into consideration that there is danger of a thing and virus infection with it difficult [to maintain a liver function over a long period of time] etc. Installation HepG2 Probably, a cell will be useful as a cell used for a biotechnology artificial liver. However, in computing biotechnology artificial hepatic clearance, it must care about that it is greatly dependent on the flow velocity which flows not only into the function which the cell itself with which it is filled up in cell culture equipment has but into a biotechnology artificial liver. for example, the hepatic clearance in a Homo sapiens liver -- about 700 ml/min 45 it s . the flow velocity in a Homo sapiens liver although this is about 100 times the value of the biotechnology artificial liver whose trial calculation was made previously -- about 1600 ml/min 40 it is -- about 100 of the flow

velocity of a biotechnology artificial liver (KIGUNASU: 15 ml/min) It is twice.
[0074] 13. in the equipment and the culture condition of the above [actual condition / artificial liver auxiliary experiment], it is the cell strain of this invention The artificial liver auxiliary experiment was conducted using CYP3 A4-GS-HepG2. The actual liver failure model was shown in drawing 6 , and the definition of effective terminal time used for evaluation of an artificial liver auxiliary system was shown in drawing 7 . Drawing 8 shows the result of the effective terminal time in each experimental group. "None of no taking a measure" forge-fire bulk here The group which is not, the thing which connects and circulated the artificial liver system in the condition that an "acellular people liver" does not have a cell, The group for which the approach by which "plasma exchange +CHDF (continuous hemofiltration)" is generally used for the therapy of liver failure, and "GS-HepG2" used the cell strain of this invention, and "Wt-HepG2" are the groups which used the wild strain (cell strain before introducing a gene). The result shown in drawing 8 shows that the outstanding prolongation-of-life effectiveness was acquired by the liver function auxiliary device of this invention. Furthermore, as shown in drawing 9 and drawing 10 , the improvement effect which was excellent in brain pressure sthenia manifestation frequency and a list in the APTT activity partial TRON boss RASUCHIN activity in a blood coagulation system, ACT activated clotting time, HPT HEPAPURASUCHIN time amount, and various parameters called the 7th factor of FVII was accepted.
[0075]
[Example 11] Nifedipine was added into the drug metabolism assay system cell which used this invention cell strain (CYP3 A4-GS-HepG2), and the nifedipine and hydroxylation object concentration of extracellular fluid were measured with time. In 100nM(s), detection of the hydroxylation object of 5% considerable amount of an addition was attained after [of addition] 5 minutes, and it increased linearly to 50% in 30 minutes. Moreover, in 60 minutes, it became 75%, and the parent drug became below detection sensitivity at this time. On the other hand in the target cell, at least 60 minutes were an average of 7%. (drawing 11) if nifedipine is added for the ketoconazole of 100microM which is 3 A4 inhibitor, cimetidine, and an erythromycin to this system after pretreatment for 10 minutes -- the amount of hydroxylation object detection of 30 minutes after -- the time of un-processing -- it became 25-35%, respectively. (Drawing 12) On the other hand, even if it used midazolam instead of nifedipine and performed hydroxylation object measurement, the amount of hydroxylation object detection became 35-45% at the time of un-adding by ketoconazole, cimetidine, and the erythromycin pretreatment. (Drawing 13) . Therefore, it is possible to evaluate simple whether the 3 A4 inhibition effectiveness is in that drug by adding a strange drug into this cell and measuring hydroxylation of nifedipine and midazolam. It is the description that metabolic turnover ability is so high that the hydroxylation object which especially flowed out into culture medium in this cell is measurable.
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TECHNICAL FIELD

[Field of the Invention] This invention relates to the liver function auxiliary device which uses the cell strain in which the transformation was carried out by the drug-metabolizing enzyme gene and the ammonia metabolic turnover enzyme gene, and this cell strain.

[Translation done.]

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TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention] The animal cell used for a hybrid mold artificial liver is roughly classified nto two. One uses the separation hepatocyte of the heterozoic origins, such as Buta. Although these have the nigh liver function, a liver function is unmaintainable for a long period of time except that cell supply takes time and effort and time amount. Moreover, since it is heterozoic, there is danger, such as immunorejection and strange virus infection. Then, what is used in the form where face to face is stood against these is HepG2. Including, it is a Homo sapiens origin cell strain. Although a liver function is low, there is a property maintainable over a long period of time that cell supply is easy. Then, we considered making some of liver functions give this Homo sapiens origin cell strain.
[0005] this invention persons are the Homo sapiens liver origin cell strains HepG2 until now. 15 which succeeded n making the removal ability of the ammonia which is one of the toxic substances give a cell. Then, by making the capacity to metabolize drugs considered to be important for a degree as a function of a biotechnology artificial liver give Homo sapiens liver origin cell strain HepG2 cell, I thought that he wanted to build the biotechnology artificial liver as an alternative removal system of a toxic substance. Therefore, construction of HepG2 cell strain which has capacity to metabolize drugs, and its functional evaluation are our purposes. Moreover, the use as a model (models, such as a toxicity test of a drug and specification of a metabolic fate) of drug metabolism research in / besides the liver failure therapy purpose / in the animal cell which has these capacity to metabolize drugs / a Homo sapiens liver] is also considered.

[Translation done.]

* NOTICES *

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2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

MEANS

[Means for Solving the Problem] By the way, a drug metabolism reaction is 16 divided roughly into the first phase reaction and the second phase reaction. Polar groups, such as a hydroxyl group, a carboxyl group, and an amino group, generate the first phase reaction by oxidization, reduction, hydrolysis, etc., or the thing of the reaction introduced is said. Although these functional groups are comparatively small polar groups compared with the second phase reaction, generally a drug loses the compatibility over a site of action by these polarization. Consequently, it becomes the form which is easy to be excreted while a pharmacological action or a physiological function falls. The second phase reaction is a reaction into which a bigger substituent than the first phase reaction is introduced. It is the conjugation reaction into which glucuronic acid, a sulfuric acid, some amino acid, a glutathione, etc. are introduced. Although there are not few compounds which have beforehand functional groups, such as a hydroxyl group, a carboxyl group, or an amino group, after these functional groups generate by the first phase reaction or being introduced, there are many compounds which undergo the second phase reaction. Since the substituent of a conjugation reaction has the polarity higher than the functional group introduced at the first phase reaction, it tends [further] to receive elimination, and it loses a pharmacological action or a physiological function.

[0007] About 80% of the drug metabolism in Homo sapiens is the cytochrome P450 which exists in a hepatic microsome. It is bearing. These P450 is bearing the first phase reaction, and consists of various subfamilies. 17 which is said for the metabolic turnover capacity of the first phase reaction to decline especially in the fulminant hepatitis patient, and is said to be especially clinically important, to occupy about 30% of the amount of manifestations in an adult liver, and to metabolize the drug of varieties also in it, and 18 that -- P450 3 A4 it is . Then, this invention person is this P450 3 A4, when evaluating the capacity to metabolize drugs in an animal cell first. It decided to observe activity. Moreover, P450 3 A4 By building an expression vector and introducing this into an animal cell, it is P450 3 A4. It tried making it discovered by the animal cell. And the possibility of the clinical application as a cell both used for a biotechnology artificial liver which performs functional evaluation about the obtained cell strain was examined, and this invention was completed.

[0008] That is, this invention relates to each following mode.

1. Cell strain in which transformation was carried out by drug-metabolizing enzyme gene and ammonia metabolic turnover enzyme gene.
2. Cell strain of one above-mentioned publication whose drug-metabolizing enzyme gene is P450.
3. Drug-metabolizing enzyme is P450. Cell strain of two above-mentioned publication which is 3 A4.
4. Cell strain of the above 1-3 given in any 1 term given ammonia metabolic turnover enzyme gene is glutamine synthetase gene.
5. Cell strain of the above 1-4 given in any 1 term given cell is the mammals animal origin.
6. Cell strain of five above-mentioned publication whose cell is the Homo sapiens liver origin.
7. Cell strain of six above-mentioned publication whose cell is the Homo sapiens hepatocyte origin.
8. Cell strain of seven above-mentioned publication whose cell is HepG2.
9. Cell strain of the above 1-8 given in any 1 term given cell is transgenics ammonia metabolic turnover Homo sapiens hepatocyte stock.
10. The cell strain of the above-mentioned above 1-8 characterized by introducing the drug-metabolizing enzyme gene and the ammonia metabolic turnover enzyme gene into the common expression vector given in any 1 term.
11. The cell strain of ten above-mentioned publication whose expression vector is a plasmid.
12. The cell strain of the above 10 or 11 publications which are the mammals cell expression vectors in which an expression vector has two or more independent multi-cloning sites and which are plasmids.

13. The cell strain of 12 above-mentioned publication which is an expression vector pBudCE4.
14. A cell strain given in the above 1 thru/or any 1 term of 13 characterized by showing the P450 activity of about 200 or more pmol/min per protein mg.
15. A cell strain given in the above 14 characterized by showing the P450 activity of about 490 or more pmol/min per protein mg.
16. A cell strain the above 14 characterized by maintaining the P450 activity of about 420 pmol/min for 80 days per protein mg, or given in 15.
17. The liver function auxiliary device which contains the cell strain of a publication in the above 1 thru/or any 1 term of 16.
18. A cell strain given in the above 1 thru/or any 1 term of 17 by which the transformation is carried out with still more nearly another drug-metabolizing enzyme gene.
19. The liver function auxiliary device which uses the cell strain of a publication for the above 1 thru/or any 1 term of 17.
20. The liver function auxiliary device which furthermore uses the cell strain of another kind and which contains the cell strain of a publication in the above 19.
21. The liver function auxiliary device of 20 above-mentioned publication whose cell strain of another kind is the Homo sapiens nonparenchymatous liver cell origin.
22. The liver function auxiliary device of the above 20 or 21 publications which are hybrid mold artificial livers.
23. A liver function auxiliary device given in the above 20 thru/or any 1 term of 22 containing a time style type culture apparatus.
24. The drug metabolism assay system which uses the cell strain of a publication for the above 1 thru/or any 1 term of 17.
25.
a) Cultivate a cell strain the above 1 thru/or given in any 1 term of 17, add b measuring object matter to a culture medium, and carry out predetermined period culture further. c) Extract a supernatant, measure the concentration of the measuring object matter in the supernatant of which d extraction was done, and the metabolized measuring object matter, respectively, and it asks for both ratio of concentration. e) The measuring object matter and the examined substance of suitable concentration are added to a culture medium, predetermined period culture is carried out further, and it is the f above-mentioned step c. It reaches. d Drug metabolism assay system which consists of measuring the effectiveness exerted on the metabolic turnover of the measuring object matter by the examined substance from change of g ratio of concentration repeatedly.
26.
a) Cultivate a cell strain the above 1 thru/or given in any 1 term of 17, add b nifedipine to a culture medium, and carry out predetermined period culture further. c) Extract a supernatant, measure the concentration of the nifedipine in the supernatant of which d extraction was done, and oxidization mold nifedipine, respectively, and it asks for both ratio of concentration. e) Nifedipine and the examined substance of suitable concentration are added to a culture medium, predetermined period culture is carried out further, and it is the f above-mentioned step c. It reaches. d Drug metabolism assay system which consists of measuring the effectiveness exerted on the oxidization mold nifedipine production by the examined substance from change of a ratio of concentration repeatedly.
27.
a) Cultivate a cell strain the above 1 thru/or given in any 1 term of 17, add b midazolam to a culture medium, and carry out predetermined period culture further. c) Extract a supernatant, measure the concentration of the midazolam in the supernatant of which d extraction was done, and oxidation type midazolam, respectively, and it asks for both ratio of concentration. e) Midazolam and the examined substance of suitable concentration are added to a culture medium, predetermined period culture is carried out further, and it is the f above-mentioned step c. It reaches. d Drug metabolism assay system which consists of measuring the effectiveness exerted on the oxidation type midazolam production by the examined substance from change of b ratio of concentration repeatedly.
[0009] As drug-metabolizing enzyme, it is P450 in the thing of arbitration well-known to this contractor, for example, the various enzymes belonging to a cytochrome P450, and a concrete target. One sort chosen from 3 A4 (CYP3 A4), CYP2C, CYP1A2, CYP2E1, CYP2D6, and CYP2A6 grade or two sorts or more can be used. In these, it is P450 3 A4. In an adult liver, the abbreviation one half of the drugs which occupy the amount of manifestations

of about 30 %, and are used by clinical can be metabolized. This P450 3 A4 P450 2D6 which metabolizes many drugs next P450 2C It is thought important to also make it discovered. It is especially P450 2D6. It is related and s observed also from the field of genetic polymorphism. This P450 2D6 The patient (PM) without activity is mportant also from a viewpoint of development of the biotechnology artificial liver used not only for the way of thinking called development of the biotechnology artificial liver which it is called 5 - 6 % by the white with 0.8 % extent although it is few, and is used for an acute liver failure patient's therapy but for the therapy of such PM by Japanese people. As an example of an ammonia metabolic turnover enzyme gene, the glutamine synthetase gene of the CHO cell origin can be mentioned, for example. From the cDNA library of marketing or public engine possession, or a vector, with cloning means, such as PCR, each of each of these genes can be easily come to hand or prepared, if it is this contractor. Moreover, the base sequence of these genes is indicated by various reference. As for the cell strain set as the object of a transformation, it is desirable that it is the mammals animal origin, for example, its cell strain of the Homo sapiens liver origin is more desirable, and it can mention HepG2 cell strain as an example of this cell strain. Such a cell can be received from various public engines (cell bank). Furthermore, it is the purposes, such as raising drug metabolism activity, and the cell strain obtained by also being able to carry out cloning of the cell strain of this invention obtained by carrying out a transformation, and carrying out cloning in this way is also the range of this invention. Although it may be supported by the different expression vector, respectively and a transformation may be performed separately, if the drug-metabolizing enzyme gene and the ammonia metabolic turnover enzyme gene are supported by the common expression vector, they are efficient and convenient. Although the vector of well-known arbitration can be used for this contractor as this expression vector, the mammals cell expression vector pBudCE4 which has two or more independent multi-cloning sites, for example, an expression vector, is suitable. In addition, the approach and means of well-known arbitration can perform easily each actuation of installation of each gene to an expression vector, the transformation of the cell strain by this expression vector, etc. by the technical field concerned. The transformation of the cell strain of this nvention may be carried out with the drug-metabolizing enzyme gene of still more nearly another class. As an example of such drug-metabolizing enzyme, it is P450. The drug-metabolizing enzyme which bears not only the first phase reaction to depend but the second phase reaction can be mentioned. For example, since it is carried out by UDP-GT (UDP-glucuronyltransfera se), glucuronide conjugation is this UDP-GT. It is thought effective to ntroduce an expression vector further. Although the liver function auxiliary device of this invention can take the configuration of well-known arbitration to this contractor, the type of the hybrid mold artificial liver containing a time style type culture apparatus is suitable. In this equipment, although it is the translation which uses the cell strain of this invention as a living thing-ingredient, the cell of other classes can also be further used as a living thing-ingredient. The drug metabolism assay system of this invention can be characterized by using the above-mentioned cell strain, and can perform it by measuring the effect of the examined substance exerted on the metabolic reaction (for example, oxidation reaction, a hydroxylation reaction) of measuring object matter (standard substance), such as nifedipine and midazolam, according [for example,] to the cell strain.

[0010] Although it ** in the example and this invention is explained in full detail hereafter, the technical range of this invention is not **(ed) at all by these.

[0011]

[Example 1] The subjects of an experiment and an approach reagent used Wako Pure Chem or the reagent chemicals of Nakarai Tesuku, unless it mentioned especially. 1 . Animal Cell Culture Approach 1.1. Host Animal Cell HepG2 Origin (Institute of Physical and Chemical Research Cell Bank RCB0459) Human Hepatocellular Carcinoma Growth Gestalt Epithelial-like [0012] 1 A .2. animal cell culture culture-medium culture medium is 0.22. muM It was used after carrying out filtration sterilization using a membrane filter (Falcon;7105). - RDF RDF (Gln+) Medium composition RDF (Gln+) (HO) powder (Japanese-made medicine) 8.44 g glucose 2.58 gNaHCO 3 2.0 g glutamine 0.333 g penicillin G 58.8 mg streptomycin 120 mgMilli-Q Water In case 1 L notes 1 use is carried out, it is 10% (vol.%) considerable-amount ****** of culture-medium capacity about fetal calf serum (fetal bovine serum;(FBS) Gibco).

notes 2 Zeocin (Invitrogen;R250-01) was added if needed. - RDF RDF (Gln-) () [Gln-] Medium composition RDF (HO) powder (Japanese-made medicine) 8.44 g glucose 2.58 gNaHCO 3 2.0 g glutamic acid 0.336 gNH 4 Cl 0.122 g penicillin G 58.8 mg streptomycin 120 mgMilli-Q Water 1 L notes 1 glutamic acid and NH 4 Cl A presentation RDF t determined to become the same number of mols as the glutamine of a culture medium (Gln+).

notes 2) RDF The (HO) culture medium is the custom-made item of Japanese-made medicine, and is the usual RDF. A glucose and a glutamine are extracted from a culture medium.

notes 3) FBS beforehand dialyzed when using it 10% (vol.%) considerable-amount ****** of culture-medium capacity.
notes 4) It is MSX (MSX (Sigma;M-5379) was added according to each MSX concentration.) to a culture medium. When needed (high MSX it is used for the acquisition experiment of a resistant strain)
notes 5) Accept the need and it is geneticin (and (Sigma (G418);G5013) Zeocin was added.) at the time of use.
- a blood serum -- medium composition NaHCO 3 0.2 g penicillin G for culture-medium blood serum culture for culture (1700 units/mg) 5.88 mg streptomycin The cellulose tube for 12 mgFBS 100 mL dialysis blood serum dialysis (Sanko Junyaku ;;Size 27/32) It dips in NaHCO 3 and 1 mMEDTA (pH 8.0) 2% (w/v), and is 10. After carrying out the autoclave between parts, It cools to 4 **, a blood serum is put into this, and it is 10 of a blood serum. The dialysing fluid of the amount of double is used and it is 30. Between parts -> 1 Time amount -> 2 Time amount -> 3 Time amount -> 4 Time amount -> over night It dialyzed.
Blood serum dialysing fluid NaCl 8.0 gKCl 0.2 gNa 2 HPO 4 and 12H 2 O 2.9 gKH 2 PO 4 0.2 g kanamycin 32 mg streptomycin 120 mg distilled water 1 L [0013] 1 As Fundamental Matter in .1.3. Animal Cell Culture, and an Actuation 1.1.3.1. Cell Culture Container Cell Culture Container 3 T-flask of a class (Sumitomo Bakelite; -- MS-20050 (area-of-base 25 cm 2, capacity 50mL) --) MS-21250 (75 cm 2, 250 mL) and MS-20800 (225 cm2, 800 mL) (A following and small T-flask, an inside T-flask, a large T-flask and an abbreviation) or 100 mm dish (Corning;25020) was used. here -- a small T-flask and 100 mm dish **** -- usually -- 10 mL a culture medium -- an inside T-flask -- 30 mL a culture medium -- a large T-flask -- 90 mL The culture medium was put in. The small T-flask was used for the subculture of the usual cell strain. Moreover, by culture experiment, it is 100 mm dish. It used.
[0014] 1 .3.2. It is 1 in principle [of a cell / passage]. Every day (checking that a cell is in a confluent condition) it carried out.)
1) The old culture medium was attracted and removed with the pipet, and 0.25% trypsin solution of the following capacity was added.
Smallness T-flask [-- It is about 10 at 30 mL2 37 **. / It cultivated between parts.] -- 5 mL, inside T-flask -- 15 mL, large T-flask
3) if a cell peels and it becomes round -- a new culture medium -- the equivalent -- in addition, pipetting was improved (since the trypsin inhibitor is usually contained into the blood serum, the reaction which strips a cell stops at this time).
4) Move cell suspension to a centrifuging tube and it is 10 at 80 xg. After carrying out part at-long-intervals alignment separation, suction removal of the supernatant was carried out with the pipet.
5) The fresh culture medium was added in the centrifuging tube, the cell was suspended, and suitable amount inoculation was carried out at new T-flask.
[0015] 0.25% trypsin solution NaCl 8 gKCl 0.2 gNa 2 PO 4 and 12H 2 O 2.9 gKH 2 PO 4 0.2 gTRYPSIN 2.5 (Difco 1:250) gMilli-Q Water 1 L [0016] 1 .3.3. In the cryopreservation of the cryopreservation method cell of a cell, a cell is suspended in the liquid which added 10% of dimethyl sulfoxide (DMSO) to the culture medium, and it is 1 mL to the Ceram tube. After having poured distributively every, putting into BICELL (Nihon Freezer) and making it freeze in -80 ** overnight, it saved in liquid nitrogen. It is 10 mL after performing defrosting in 37-degree C warm water and melting a culture medium. Cell suspension is moved to the centrifuging tube into which the culture medium was put, and it is 10 at 80 xg. After carrying out part at-long-intervals alignment separation, suction removal of the supernatant was carried out with the pipet, and the cell was inoculated into new T-flask.
[0017] 1 .3.4. . Which Measured Cell Density Measurement Approach (Measurement by Dye Exclusion Assay) 19 Viable Cell, and Total Cell Concentration Using Dye Exclusion Assay Which Used Trypan Blue -- this -- 0.2% (W/v) of Trypan Blue Water Solutions, and NaCl 4.25% (W/v) of Water Solutions 4:1 (one drop (about 15 mu L) (it carries)) It mixes at a rate, equivalent mixing of the cell suspension is carried out at this liquid, and it is Burker-Turk. Mold counting chamber (ERMA 4296) In . counting chamber which is the approach of carrying out a speculum immediately and measuring viable cell concentration and total cell concentration, the cell concerning two sides, right-hand side and the bottom, was excluded at the time of measurement. 20 which amended the depth of a counting chamber according to the assay value of counting chamber attachment, calculated the volume on a counting chamber, and computed cell concentration here.
[0018] 2 .HPLC Used Drug Metabolism Activity (P450 3 A4 Activity) System-of-Measurement 2.1.HPLC The Used Density Measurement Approach 21 of Substrate and Metabolite - 24 Drug-Metabolism Activity (P450 3 A4 Activity) P450 3 A4 The hormone matter testosterone (the first chemicals; UC-339, molecular weight ; 288.4)

metabolized specifically is made into a substrate. 6 which is metabolite Beta-hydroxytestosterone (the first chemicals ;;UC-282, molecular weight ; 304.4) HPLC It evaluated by measuring. HPLC Internal standardization was used as the quantum approach at the time of carrying out. androstenedione (4-Androstene-3, 17-dione) (first chemicals ;;UC-300) was used as internal standard matter here. As introduction standard concentration, they are a substrate, metabolite, and each internal standard matter 100microM. It prepares so that it may become, and it is HPLC. The concentration of metabolite was computed in the substrate list after that based on the peak area of a chart.

[0019] - HPLC Conditions HPLC Equipment -- The Shimadzu liquid chromatograph system ; LC10AD;
Column used -- C 18 Inertsil ODS-3V which are a column; (4.6 x150 mm, 5 mu m, GL Sciences Inc.)
** ** buffer -- A Liquid methanol: Distilled water =45 : 55 B Liquid methanol: Distilled water =90 : 10.
Buffer It sets to production and is HPLC. After mixing the ** methanol (Nakarai Tesuku) and distilled water by each ratio, degassing was performed suitably.
** ** -- 1.0 ml/min;
Column temperature -- Room temperature;
** Constant wavelength -- 254 nm;
P450 3 A4 HPLC gradient in activity Conditions [0020]
Table 1]

時間(分)	A液	B液
0	100%	0%
↓	↓	↓
10	0%	100%
~12	0%	100%
12~	100%	0%

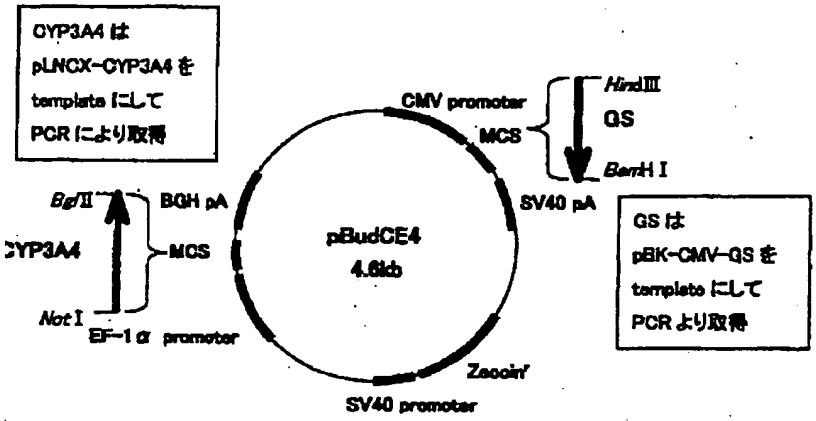
[0021] 2 Quantum P450 3 A4 of the amount of .2. cell total protein Generally activity is pmol 6. Beta-hydroxytestosterone formed/min/mgprotein It is expressed in the unit to say. In case drug metabolism activity is searched for using cell culture supernatant liquid, the amount of cell total protein (mg protein) must be measured. Here, we decided to measure the amount of cell total protein using BCA Protein Assay Reagent Kit (Pierce;23225). The quantum approach followed the attached manual. The adjustment procedure of the cell crushing liquid (crude enzyme liquid) in the amount measurement of cell total protein is shown below. A procedure is shown below.
1) a cell -- the culture medium (in the case of 100 mm dish culture, culture-medium 5 ml is a standard) of a known amount -- suspending -- 500 of them mul a centrifugal tube -- moving -- 1000 xg 10 a part -- between -- centrifugal separation was carried out by 4 **.
2) supernatant liquid -- removing -- 100 mM potassium phosphate buffer (pH7.4) -- suspending -- 1000xg -- 10 a part -- between -- centrifugal separation was carried out by 4 **.
3) By the ultrasonic fungus body destructor (marine electrical machinery T -- A-4200), it is 5. After the sonication during a second, and 1 It ice-cools between parts and is this 4 **********.
4) 14000 xg 10 The quantum of the amount of cell total protein was carried out after centrifugal separation between parts using supernatant liquid by 4 **.

[0022] 2 Pretreatment 25HPLC of .3. cell culture supernatant liquid (sample) It is in charge of using and measuring the substrate and metabolite concentration in cell culture supernatant liquid, and is column Sep-Pak Plus C 18 (Waters) for sample pretreatment. It used and the sample was refined. A procedure is shown below. It pours into a column. (apply) The rate of flow at the time of carrying out is 2 - 4 ml/min. It took care so that it might become.
1) -- 100% methanol 2 ml a column -- 2 Time apply It carried out.
2) -- distilled water 2 ml a column -- 2 Time apply It carried out.
3) -- a sample -- 2 ml and apply It carried out.
4) -- distilled water 2 ml a column -- apply It carried out and the column was washed.
5) -- 100% methanol 2 ml apply It carried out and the quality of the specified substance was eluted.

[0023] 3 P450 3 A4 in . animal cell (intact cell) The activity measurement approach 21 and 23110 mL a culture medium RDF (Gln+ or Gln-) -- using -- 100 mm dish a cell -- 1 x10 7 an individual -- seeding was carried out. Culture is the basis of 5 %CO 2 and 37 **, and 16. It carried out time.
2) Concentration is testosterone (it melted to the methanol 100% so that it might be set to 100mM) which culture-medium exchange is performed (amount of culture media 10 mL), and is a substrate 100 mu M It added so that it

night become.
3) It is 2 at 5 %CO 2 and 37 **. This culture supernatant 2 mL after carrying out time amount culture It used and activity was measured.
4) After culture termination, the cell was removed by trypsinization and the amount of cell total protein was measured.
[0024]
[Example 2] 4 . immanency P450 3 A4 Homo sapiens liver drug-metabolizing enzyme induction medicine rifampicin for the improvement in activity It hits applying an addition culture animal cell to a biotechnology artificial liver, and s a wild strain HepG2. A cell and gene recombination ammonia metabolic turnover HepG2 built from this invention person's until now P450 3 A4 of the internality in a cell (GS-HepG2 a cell and MSX300 muM resistant strain) improvement in activity was tried. until now -- HepG2 a cell -- setting -- dexamethasone, phenobarbital, 3-methylcholanthrene, prednisolone, carbamazepine, and rifampicin etc. -- 26, 27, and 28 to which it is reported that capacity to metabolize drugs rises by drug induction if drugs are added to a culture medium. Then, in these drug nducer, it is CYP3A. rifampicin made the most effective as an inducer By adding, it is internality P4503 A4. improvement in activity was aimed at.
1) 100 mm dish RDF Finally it is 10 mL about a culture medium (FBS is included 10%). It added so that it might become.
2) Wild strain HepG2 It is transgenics GS-HepG2 to a list. About a cell (HNAA-300A cell strain), it is 1 x10 7cells. Seeding was carried out.
3) 37 ** and 5%CO 2 Basis 12 It cultivated time.
4) Culture-medium exchange. 10%FBS Or it contains, it is RDF of a non-blood serum. It is 10 mL about a culture medium. It added.
5) 37 ** and basis 24 of 5%CO 2 It cultivated time.
6) Culture-medium exchange. rifampicin melted to dimethylsulfoxide (DMSO) The last concentration is 10 and 50,100,200,300,500. It is RDF of a non-blood serum so that it may be set to mu M. It adds to a culture medium. It s the non-blood serum RDF as control. It is DMSO to a culture medium. The chisel (0.1%) was added.
7) 37 ** and basis 24 of 5% CO2 After carrying out time amount culture, culture-medium exchange and rifampicin of each concentration were added.
8) Perform 7 for six days (6 culture-medium exchange was performed every day during a day), and it is 200 after that. mu M testosterone It adds to a culture medium and is P450 3 A4. Activity was measured.
[0025] The above-mentioned wild strain HepG2 Homo sapiens liver drug-metabolizing enzyme induction medicine rifampicin to a cell The result obtained in the addition culture experiment was shown in drawing 1 . This is FBS in a culture medium. rifampicin when it does not contain with the case where it contains Wild strain HepG2 in each concentration P450 3 A4 of a cell Activity is shown. Wild strain HepG2 P450 3 A4 of a cell Activity is 0.6 pmol/min/mg-protein in the condition of not applying drug induction. The value to say was shown. Moreover, it is P450 3 A4 most. It is FBS that whose activity was high. It sets to the included culture medium and is rifampicin 100. muM It is that to which induction was applied and an activity value is 2.3 pmol/min/mg-protein. It became. It s FBS in a culture medium. By comparing the case where it does not contain with the case where it contains, it is FBS. It sets to the culture to include and is internality P450 3 A4. It turned out that it is easy to be guided. FBS important various factors including a growth factor are contained in inside (blood serum). Therefore, FBS By nothing culture, since the important factor for surviving was drained, I thought that it was because the response to change of the external world in a cell becomes blunt.
[0026] Furthermore, gene recombination ammonia metabolic turnover HepG2 Homo sapiens liver drug-metabolizing enzyme induction medicine rifampicin received a cell (GS-HepG2 a cell, MSX300 muM resistant strain) The result obtained in the addition culture experiment was shown in drawing 2 . This is each [when it does not contain with the case where a dialysis blood serum is included] rifampicin. HNAA-300A in concentration P450 3 A4 of a cell strain It is activity. In the condition of not applying drug induction as shown in this drawing, it is HNAA-300A. The cell strain had the almost same activity as wild strain HepG2 cell. However, rifampicin By the drug induction by addition, it is a wild strain HepG2. It compares with a cell and is P450 3 A4. It was checked that induction is hard to be carried out. HNAA-300A A cell strain is a wild strain HepG2. It is pBK-CMV-GS to a cell. MSX after introducing a vector And G418 It uses and selection is applied. It is HNAA-300A if it thinks including the ability to survive also under such a harsh environment. Since the susceptibility over the external world of a cell strain is blunt, it is a wild strain HepG2. It compares with a cell and is rifampicin. P450 3 A4 to depend I thought that it was

what induction cannot take place to easily.
[0027]
[Example 3] 5 .P450 3 A4 Construction 5.1.P450 3 A4 of an expression vector Expression vector (pBudCE-GS-CYP3 A4) As an outline 5.1.1. use plasmid use plasmid of construction, it is pBudCE4 29 (Invitrogen;V532-20). It used. This is multicloning site (MCS) 2 KA possession is carried out and it is each MCS. It is humancytomegalovirus immediate-early promoter (CMV) to the upstream. And human elongation 1 Alpha-subsuit (EF-1 alpha) promoter It has. As a marker gene, it is Zeocin at Escherichia coli and an animal cell. It is selectable. P450 3 A4 The outline of construction of an expression vector is shown in the following table 2.
[0028]
[Table 2]



[0029] 5 .1.2. Preparation 5.1.2.1. Use Strain Escherichia Coli TOP10 [(Invitrogen;C 615-00)0030] of Use Plasmid 5 .1.2.2. Culture Medium (LB-Zeocin)
Deionized water 100 mL Bacto-tryptone 1 (Difco) g, Bacto yeast extract0.5 (Difco) g, and NaCl 0.5 g pH 7.0 after melting The autoclave was prepared and carried out. It is 2 g when making it a plate. Agar It added. It is Zeocin if it becomes at least 55 ** after an autoclave. 50 mug/mL It added so that it might become.
[0031] 5 the creation 1 of the transformation and competent cel of the Escherichia coli by the .1.2.3. calcium chloride method -- the Escherichia coli which carries out a transformation -- 5 mL LB Shaking culture was carried out by 37 ** by the culture medium overnight.
2) -- preculture liquid 2 mL 40 mL LB a culture medium -- inoculation -- carrying out -- 37 **2 Time amount culture was carried out.
3) -- the inside of ice -- ten a part -- the above -- leaving it -- 4 **, 6000 xg, and 5 The harvest was carried out by carrying out part at-long-intervals alignment separation.
4) -- 50 mM CaCl 2 20 mL which ice-cooled precipitation suspending -- 0 **20 It was left between parts.
5) -- centrifugal separation -- after a harvest and 50 mM CaCl 2 4 mL suspending -- 200 muL It poured distributively every.
- Transformation 200 muL It is DNA to a competent cel. A solution is added a suitable amount (0.01 mug extent), and it is 1 in ice. Time amount neglect was carried out. 42 At **, it is 90. After giving the heat shock during a second, it quenches by iced water, and it is 0.8 mL. LB A culture medium is added and it is 1 at 37 **. Time amount shaking culture was carried out. 100 among this culture medium muL LB-Zeocin It extended to the culture medium and cultivated by 37 ** overnight.
[0032] 5 Plasmid DNA from .1.2.4. Escherichia Coli Preparation (Alkali Extraction Method)
5 mL LB-Ampicilin Inoculation of the Escherichia coli transformant is carried out to a liquid medium, and it is 16 at 37 **. Time amount shaking culture was carried out. It is 1.5 mL about culture medium. Extent picking, 12000 xg, and 2 Part at-long-intervals alignment separation was carried out, and the harvest was carried out. solution I100 after stirring precipitation with a vortex mixer It suspends completely in muL and is solution II200. muL It is 3-4 quietly moreover. Time fall mixing is carried out and it is 5 correctly in ice. It was left between parts. It is solution III cooled beforehand 150 muL In addition, it mixes violently and is 5 in ice. It was left between parts. 12000 xg and 5 After carrying out part at-long-intervals alignment separation, supernatant liquid was moved to a new sample tube. 20 after performing a phenol-chloroform extraction and performing ethanol precipitation further about this supernatant liquid mug/mL Dnase free RNase TE 50 included muL It dissolved.
solution I: 50 mM Glucose, 25 mM Tris--Cl (pH 8.0), 10 mM EDTAsolution II: 0.2 N NaOH and 1%%SDS (it prepares

at the time of an important point)
solution III: 5 M Acetic-acid water solution containing potassium acetate [0033] 5 .2. gene relation -- fundamental -- actuation 5.2.1. agarose electrophoresis 1/2 xTAE It carried out by 0.8% agarose gel (TaKaRa;Agarose LO3) using the buffer solution. The small electrophoresis tub (Advance Co.Ltd.;Mupid2) was used as a migration tub. A sample is usually DNA. It is 1/10 to a solution. xten of an amount The stain solution for migration was added and produced. Migration is constant-voltage 100 V. 40 The part was performed. They are after migration and gel with an ethidium bromide water solution (EtBr) (0.5 mu g/ml) 10 It dyes between parts and is DNA at Tran Swi Rumi Noether (Ultra Violet C62). The band was observed. A photograph is UV. A photograph was taken with the Polaroid (trademark) camera using the filter and the red filter.
[0034] 5 .2.2. ethanol precipitate DNA To the included solution, it is 1/10. 3 M of an amount A sodium acetate solution (pH 5.2) is added and it is 2.5 of the solution further. The 100 % ethanol of the amount of double is added, and it mixes, and is 20 at a room temperature. It was part-left. It is this 18000 xg and 10 Part at-long-intervals alignment separation was carried out, and supernatant liquid was removed. It is 70% ethanol to this precipitate Optimum dose ****, 18000 xg, and 10 After carrying out part at-long-intervals alignment separation, supernatant liquid is removed, and they are the sterilized water of after reduced pressure hardening by drying and optimum dose, or Sterilization TE about precipitate. It dissolved in the buffer solution (10 mM Tris-Cl (pH 7.5) and 1 mMEDTA).
[0035] 5 .2.3. phenol chloroform-extraction DNA It is TE about a solution. It was saturated with the buffer solution and phenol chloroform isoamyl alcohol (25:24:1) mixed liquor was mixed equivalence, in addition well. this -- 18000 xg -- at-long-intervals alignment separation was carried out for 10 minutes, and the upper layer was moved to a new sample tube.
[0036] 5 .3.PCR P450 3 A4 to depend A gene and GS Preparation / use device GeneAmpR PCR System 2400 and (PERKIN ELMER) templateP450 3 A4 of a gene About a gene, they are pLNCX-CYP3 A4 (Els M.De Grone it presents from a teacher), and GS. About a gene, it is PBK-CMV-GS. It used. Used primer is shown in the following table 3.
[0037]
[Table 3]

GS遺伝子をPCRにより獲得する際に用いたプライマー

N末端側 *HindIII*
5' - AAAAAAAAGCTTACCATGGCCACCTCAGCAAGTTCCC - 3'

C末端側 *BamH I*
5' - CCCCCCGGATCCAATTAGTTTTTGTATTGGAAGGGCTC - 3'

CYP3A4をPCRにより獲得する際に用いたプライマー

N末端側 *Not I*
5' - TTTTTTGCGGCCGCGTGATGGCTCTCATCCCAGACTTGG - 3'

C末端側 *BglII*
5' - GGGGGGAGATCTATTCAGGCTCCACTTACGGTGCCATC - 3'

[0038] In addition, it is Kozak so that it may be in charge of the design of a primer and the translation in an animal cell may be started more correctly. It referred to the array (A/G NN ATG G). Moreover, it is each PCR to the following table 4. A presentation and conditions of a reaction were shown.
[0039]
[Table 4]

primer GS *Hind*III (20 pmol/μl)	1 μl
primer GS *Bam*H I (20 pmol/μl)	1 μl
Template (pBK-CMV-GS) (50 pmol/μl)	1 μl
KOD polymerase buffer (×10)	5 μl
dNTP 2 mM	5 μl
$MgCl_2$ 25 mM	2 μl
KOD polymerase 2.5 unit/μl	1 μl
滅菌水	34 μl
total	50 μl

primer CYP3A4 *Not* I (20 pmol/μl)	1 μl
primer CYP3A4 *Bgl*II (20 pmol/μl)	1 μl
Template (pLNCX-CYP3A4) (50 pmol/μl)	1 μl
KOD polymerase buffer (×10)	5 μl
dNTP 2 mM	5 μl
$MgCl_2$ 25 mM	2 μl
KOD polymerase 2.5 unit/μl	1 μl
滅菌水	34 μl
total	50 μl

PCR条件

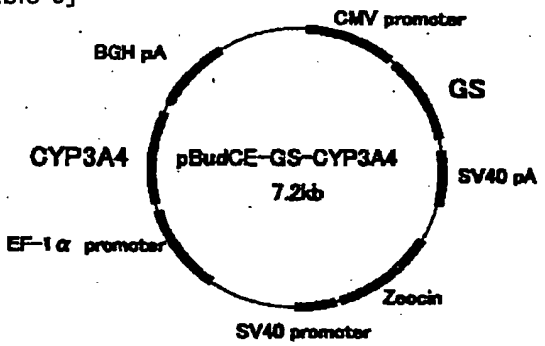
98℃ 15 sec → 65℃ 2 sec → 74℃ 30 sec

← 25 cycles →

[0040] 5 .4.pBudCE4 P450 3 A4 which is a vector (invitrogen) and an insertion A gene and GS pBudCE4 prepared by ligation5.1.2.4. with a gene The P450 3 A4 gene and GS which were prepared by the vector and 5.3. It decided to carry out ligation using a gene. This process is TAKARA SHUZO CO., LTD. An order was placed with the gene-analysis pin center,large. Consequently, obtained P450 3 A4 Expression vector (pBudCE-GS-CYP3 A4) It is shown in the following table 5. This vector is pBudCE4. To two multicloning site of a vector, it is pLNCX-CYP3 A4. CYP3 A4 (gene of P450 3 A4) and pBK-CMV-GS of the origin GS of the origin A gene is inserted. .

[0041]

[Table 5]



[0042] Furthermore, 5.3. is followed and it is P450 3 A4. A gene and GS It is PCR about a gene. It amplifies and is PCR. Product 4 muL Electrophoresis was carried out using 0.8 % agarose gel. The result is shown in drawing 2 . Rain ** is P450 3 A4. Gene (about 1.5 kbp(s)) and rain ** is GS. Gene (about 1.1 kbp(s)) and rain ** is a lambda/Hind III marker. P450 3 A4 which is the target gene from this A gene and GS Acquisition of a gene was checked. P450 3 A4 P450 3 A4 inserted in the expression vector The sequence result of a gene (CYP 3 A4) is shown in the following table 6. Di CYP3 A4 registered into - TABESU Base sequence 33 When it compares, it is 6 after an initiation codon (ATG). The base eye was not C (cytosine) but A (adenine). However, from configuration amino acid serving as a leucine with both (CTC and CTA), in case it is made to translate into protein, it is thought that P450 3 A4 inserted in a vector has original activity.

[0043]

[Table 6]

(N末端側)TCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCA *Not I* 開始コドン
AAGTTTTTTTCTTCCATTTCAGGTGTCGTGAACACGTGGTCGGGGCCGCGTGATGGCTGT
AATCCCAGACTTGGCCATGGAAACCTGGCTTCTCCTGGCTGTCAGCCTGGTGCTCCTCTA
TCTATATGGAACCCATTCACATGGACTTTTTAAGAAGCTTGGAATTCCAGGGCCCACACC
TGTGCCTTTTTTTGGGAAATATTTTGTCCTACCATAAGGGCTTTTGTATGTTTGACATGGA
ATGTCATAAAAAGTATGGAAAAGTGTGGGGCTTTTATGATGGTCAACAGCCTGTGCTGGC
TATCACAGATCCTGACATGATCAAAACAGTGCTAGTGAAAGAATGTTATTCTGTGTTCAC
AAACGGGAGGCCTTTTGGTCCAGTGGGATTTATGAAAAGTGCCATCTCTATAGCTGAGGA
TGAAGAATGGAAGAGATTACGATCATTGCTGTCTCCAACCTTCAACAGTGGAAAACTCAA
GGAGATGGTCCGTATCATTGCCCAGTATGGAGATGTGTTGGTGAGAAATCTGAGGCGGGA
AGCAGAGAAGAGGCAAGCCTGTCACCTTGAAAGACGTCTTTGGGGCCTACAGCATGGATGT
GATCACTAGCACATCATTTGGAGTGAACATCGACTCTCTCAACAATCCACAAGACCCCTT
TGTGGAAAACACCAAGAAGCTTTTAAGATTTGATTTTTTGGATCCATTCTTTCTCTCAAT
AACAGTCTTTCCATTCCTCATCCCAATTCTTGAAGTATTAAATATCTGTGTGTTTCCAAG
AGAAGTTACAAATTTTTTAAGAAAATCTGTAAAAAGGATGAAAGAAAGTCGCCTCGAAGA
TACACAAAAGCACCGAGTGGATTTCCTTCAGCTGATGATTGACTCTCAGAATTCAAAAGA
AACTGAGTCCGACAAAGCTCTGTCCGATCTGGAGCTGGTGGCCGAATCAATTATCTTTAT
TTTTGCTGGCTATGAAACCACGAGCAGTGTTCTCTCCTTCATTATGTATGAACTGGCCAC
TCACCCTGATGTCCAGCAGAAACTGCAGGAGGAAATTGATGCAGTTTTACCCAATAAGGC
AGCACCCACCTATGATACTGTGCTACAGATGGAGTATCTTGACATGGTGGTGAATGAAAC
GCTCAGATTATTCCCAATTGCTATGAGACTTGAGAGGGTCTGCAAAAAAGATGTTGAGAT
CAATGGGATGTTCATTCCCAAAGGGTGGGTGGTGATGATTCCAAGCTATGCTCTTCACCG
TGACCCAAAGTACTGGACAGAGCCTGAGAAGTTCCTCCCTGAAAGATTCAGCAAGAAGAA
CAAGGACAACATAGATCCTTACATATACACACCGTTTGGAAGTGGACCCAGAAACTGCAT
TGGCATGAGGTTTGCTCTGATGAACATGAAACTTGCTCTAATCAGAGTCCTTCAGAAGTT
CTCCTTCAAACCTTGTAAAGAAACACAGATCCCCCTGAAATTAAGCTTAGGAGGACTTCT
TCAACCAGAAAAACCCGTTGTTCTAAAGGTTGAGTCAAGGGATGGCACCGTAAGTGGAGC
終止コドン
Bgl II
CTGAATAGATCTGGCCGGCTGGGCCCGTTTCGAAGGTAAGCCTATCCCTAACCCTCTCCT
CGGTCTCGATTCTACGCGTACC

[0044] Moreover, P450 3 A4 GS inserted in the expression vector The sequence result of a gene is shown in the following table 7. GS About a gene, it is template. pBK-CMV-GS carried out GS Array 30 of a gene It was completely the same. pBK-CMV-GS Introduced CHO A cell (CN9-500-4 cell strain) and HepG2 It sets into a cell (HNAA-300A cell strain), and is GS. GS inserted in this vector from activity being checked It is thought that it has the function as original protein.

[0045]

[Table 7]

(N末端側)TCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATA
GGGAGACCC*AAGCTT*ACCATGGCCACCTCAGCAAGTTCCCAGTTGAACAAAAACATCAAG
Hind III 開始コドン
CAAATGTACCTGTGCCTGCCCAAGGGTGAGAAAGTCGAAGCCATGTATATCTGGGTTGAT
GGTACTGGAGAAGGACTGCGCTGCAAAACCCGCACCCTGGACTGTGAGCCCAAGTGTGTA
GAAGAGTTACCTGAGTGGAATTTTGATGGCTCTAGTACGTTTCAGTGTGAGGGCTCCAAC
AGTGACATGTATCTCAGCCCTGTTGCCATGTTTCGGGACCCCTTCCGCAGAGATCCCAAC
AAGCTGGTGTTCTGTGAAGTTTTCAAGTACAACCGGAAGCCTGCAGAGACCAATTTAAGG
CACTCGTGTAAACGGATAATGGACATGGTGAGCAACCAGCACCCCTGGTTTGGAATGGAA
CAGGAGTATACTCTGATGGGAACAGATGGGCACCGTTTTGGTTGGCCTTCCAATGGCTTT
CCTGGGCCCCAAGGTCCGTATTACTGTGGTGTGGGCGCAGACAAAGCCTATGGCAGGGAT
ATCGTGGAGGCTCACTACCGCGCCTGCTTGTATGCTGGGGTCAAGATTACAGGAACAAAT
GCTGAGGTCATGCCTGCCCAGTGGGAATTCCAAATAGGACCCTGTGAAGGAATCCGCATG
GGAGATGATCTCTGGGTGGCCCGTTTCATCTTGCATCGAGTATGTGAAGAGTTTGGGGTA
ATAGGAACCTTTGACCCCAAGCCCATTCCTGCGAACTGGAATGGTGCAGGCTGCCATACC
AACTTTAGGACCAAGGCCATGCGGGAGGAGAATGGTCTGAAGGACATCGAGGAGGCCATC
GAGAAACTAAGCAAGCGGCACCGGTACCACATTCGAGCCTACGATCCCAAGGGGGGCCTG
GACAATGCCCGTCGTCTGACTGGGTTCCACGAAACGTCCAACATCAACGACTTTTCTGCT
GGTGTCGCCAATCGCAGTGCCAGCATCCGGCATTCCCCGGACTGTCGGCCAGGAGAAGAA
GGTTACTTTGAAGACCGCCGCCCTCTGCCAATTGTGACCCGTTTGCAGTGACAGAAGCC
ATCGTCCGCACATGCGTTCTCAATGAGACTGGCGACGAGCCGTTCCAATACAAAAACTAA
TT*GGATCC*GAACAAAAACTCATCTCAGAAGAGGATCTGAATATGCA (C末端側) 終止コドン
BamH I

[0046]
[Example 4] 6 .P450 3 A4 Introductory P450 3 A4 to the animal cell of an expression vector It is a wild strain HepG2 about an expression vector. Cell (ATCC No.HB-8065) And gene recombination ammonia metabolic turnover HepG2 already built by this invention person It tried to introduce into a cell (HNAA-300 A share). It is P450 3 A4 to an animal cell. In introducing an expression vector, this vector must be prepared by the high grade. Then, Wizard PureFection Plasmid DNA Purification System (A2160; how to make DNA stick to a magnet and to collect plasmid DNA s of a high grade after removing endotoxin etc.) was used. The approach followed the attached manual (the publication of an approach is omitted). P450 3 A4 of the high grade obtained above An expression vector is used and it is transfection. It carried out. transfection The approach used the liposome method (TaKaRa and Trans IT Polyamine Transfection Reagents).
1) It is 35 mm dish about a 4 x10 5 piece cell. It cultivated by seeding and 37 ** overnight. As a culture medium, they are HepG2 and CHO-K1. It sets and is FBS. About an entering RDF (Gln+) culture medium, they are HNAA-300A and CN 9-500-4. It set and the RDF (Gln-) culture medium containing a dialysis blood serum was used.
2) The next day and 100 mu l It is TransIT Transfection Reagent to a serum free medium. 20 mu l It is vortex moreover. It mixed.
3) It is 10 min at a room temperature, It cultivated.
4) It is vector DNA 3 to this. mu g It is pipetting quietly moreover. It carried out.
5) It is 10 min at a room temperature, It cultivated.
6) What was prepared by 5 was added with POTAPOTA to the cell culture medium prepared by 1. dish It shook slowly and was made to mix.
7) 72 It cultivated by time amount and 37 **.
8) Carry out trypsinization of what was prepared by 7, remove it, and it is smallness about the whole quantity. - T Seeding was carried out to the flask (area-of-base 25cm 2). A culture medium is Zeocin. The passage was carried out using the RDF (Gln+ and Gln-) culture medium which is not included.
9) Smallness - T Zeocin after becoming confluent in a flask It planted in the included selective medium and inherited. Zeocin in a selective medium concentration -- HepG2 And HNAA-300A **** -- 200 mug/ml ** -- it carried out.
[0047] In this way, obtained P4503 A4 HepG2 made to discover Cell (CYP3 A4-GS-HepG2) P450 3 A4 The result of having measured activity is shown in Table 8. in addition, in front Naka, "P450 3 A4-HepG2" is written (the inside of each table of a following and book specification -- the same). [this cell]
[0048]
[Table 8]

細胞株	P450 3A4 活性 (testosterone 6β-hydroxylation 活性 pmol/min/mg-protein)
P450 3A4-HepG2	490
HepG2(薬物誘導なし)	0.6
HepG2(薬物誘導あり)	2.3

[0049] clear from Table 8 -- as -- P450 3 A4 Installation HepG2 the activity value in a cell -- about 800 of a wild strain twice -- a high value and 490 pmol/min/mg-protein It was shown. P450 3 A4 CHO made to discover even if it compares with the activity in a cell (P450 3 A4-CHO cell strain) -- P4503 A4-HepG2 the activity in a cell strain -- about 20 twice -- it means that the high value was shown P450 3 A4 Installation CHO It compares with a cell and is P450 3 A4. Installation HepG2 P450 3 A4 in a cell Activity is 20. Although it became high more than twice This is an environment (for example, it sets for oxidation / reduction reaction by P450) which supports the reaction mechanism of CHO. It compares with a cell and is HepG2. It sets into a cell and is P450. P450 P450 from a reductase It is thought that it is because it remains [that electronic supply is performed actively etc. and] in intracellular.
[0050] It is 24 here. P450 3 A4 of the first hepatocyte which carried out time amount culture An activity value is 252.8 pmol/min/mg-protein. 35 reported. Moreover, at the thing using rat founder hepatocyte, it is P450 3 A4 after 4-hour culture. Activity is 407 pmol/min/mg-protein and 24. The activity after time amount culture is 158

pmol/min/mg-protein. 36 which has become. It is P450 3 A4 from this. Installation HepG2 It can be said that the cell strain had the first hepatocyte, an EQC, or the activity beyond it. However, in vivo P450 3 A4 of the Homo sapiens hepatocyte which can be set For activity, individual difference is 1000 of a certain thing - 1500 pmol/min/mg-protein. It is called extent, turns to 37 and the clinical application to Homo sapiens, and is P450 3 A4-HepG2 further. P450 3 A4 of a cell I thought that he wanted to raise activity further.

[0051]

[Example 5] 7 .P450 3 A4 GS in a manifestation animal cell Gene amplification approach P450 3 A4 using a genetic system P450 3 A4 in a manifestation animal cell It is GS in order to raise activity. Gene amplification in a genetic system was performed.

1) -- Smallness-T a flask (area-of-base 25 cm 2) -- setting -- HepG2 a cell -- 200 mug/mlZeocin And MSX of each concentration the included selective medium -- a cell -- about -- 5 x10 five-piece seeding was carried out, and culture-medium exchange was performed until it became confluent.

2) -- the selective medium which the cell was exfoliated [selective medium] when becoming confluent, and raised MSX concentration (Methionine Sulfoximine) -- about 5x105 The stroke of carrying out individual seeding was repeated and the gene amplification in a hetero condition was tried.

[0052] Consequently, P450 3 A4 Installation HepG2 It sets into a cell and is each MSX. The resistant strain of concentration was acquired. Next, it is P450 3 A4 about each resistant strain. Activity was measured (3). The result is shown in drawing 3 . In addition, MSX P450 3 A4 Since activity may have been affected, in the passage before activity measurement, MSX was not added to a culture medium. Moreover, it is MSX also to the culture medium at the time of carrying out activity measurement. It did not add. MSX It is GS by addition. P450 3 A4 using a gene amplification system Although improvement in activity was aimed at, the almost same activity value was shown also in which resistant strain. From this, it is GS. The gene amplification in a gene is used and it is P450 3 A4. It can be said that activity was not able to be raised. Each cell strain is MSX why here. In spite of having acquired resistance, it considered as follows that P450 3 A4 activity did not rise.

1) P450 3 A4 GS introduced into the expression vector Since it is not by the gene and gene amplification happened in GS gene of internality, it is P450 3 A4. Activity did not rise (P450 3 A4 activity is change nothing and GS in this case activity will rise).

2) P450 3 A4 GS introduced into the expression vector Although gene amplification happened in the gene, it is P450 3 A4 at a certain factor. A gene did not amplify (P450 3 A4 activity is change nothing and GS in this case activity rises).

3) GS The gene amplification in a genetic system itself had not worked (it is P450 3 A4 in this case activity or GS activity does not change, either). That is, by devices other than gene amplification (for example, film variation etc.), it is MSX. It is thought that resistance was acquired.

[0053]

[Example 6] 8 .P450 3 A4 Installation HepG2 GS in a cell the activity measurement method 32 -- in order to verify these things -- each MSX Concentration resistance P450 3 A4 Installation HepG2 GS of a cell It decided to evaluate activity.

8 .1.GS Activity measurement principle GS Gamma-glutamyl The catalyst of the transition reaction is carried out. namely, hydroxylamine from -- gamma-glutamylhydroxyamate generating -- this gamma-glutamylhydroxyamate ferric chloride Addition shows characteristic brown. This is hydroxylamine. Used glutamine synthetase It is the principle of activity measurement. This reaction is used and it is GS. Activity was measured.

[0054] 8 .2.GS He is TBS about activity measurement approach 1 cell suspension. 500 once washing muL Imidazole buffer It suspended.

2) 10% beta-mercaptoethanol (.) 5 muL It added. (antioxidant)

3) an ultrasonic fungus body destructor (T-A-4200; marine electrical machinery incorporated company) -- 10 After the sonication during a second, and 1 a part -- between -- ice-cooling -- 3 ********** -- the cell was crushed by things.

4) 10% beta-mercaptoethanol It is 5 again. muL It added.

5) It is 100 mM phenylmethylsulfonylfluoride (PMSF; ethanol solution) 5 muL It added.

6) It is 200 mM Pepstatin A (ethanol solution) 5 muL It added. (PMSF and Pepstatin A are protease inhibitors)

7) 18,000xg.5 Centrifugal separation was carried out by 4 ** between parts.

8) Supernatant liquid was moved to new EPPEN and the volume was measured.

9) It is 15 in 37 ** correctly [after adding and carrying out the vortex of the reaction substrate liquid to crude

enzyme liquid]. It was made to react between parts.
10) 0.75 mL FeCl3 solution is added and it is 5 at 18,000 xg. After carrying out part at-long-intervals alignment separation, supernatant liquid is struck to a cuvette, and it is A 535. The absorbance was measured. 11) Measured value to blank The value was lengthened and activity was searched for. the bottom of this Measuring condition -- A 535 =0.340 the time -- 1 unit ** -- 32 carried out. It is gamma-glutamyl transfer to Table 9. About the presentation of an activity measurement reagent, it is FeCl 3 in Table 10. The presentation of a solution was shown.

[0055]

[Table 9]

	Stock液の組成	測定時における添加量
Imidazole-HCl	100 mM, pH7.2	250 μL
$MnCl_2$	125 mM	20 μL
L-Glutamine	250 mM	100 μL
arcenate	200 mM, pH7.2	50 μL
Hydroxylamine	1 M, pH7.2	50 μL
ADP·Na	2.5 mM	20 μL
酵素液		10 μL
Total volume		500 μL

[0056]

[Table 10]

	Stock液の濃度	測定時における添加量
$FeCl_3$	1.11 M	250 μL
HCl	2.01 M	250 μL
Trichloroacetic acid	0.60 M	250 μL
Total volume		750 μL

[0057] Moreover, GS Since it is the purpose to measure activity, it is GS. Extinction values other than an enzyme reaction should be removed. Then, blank as shown in Table 11 It took.

[0058]

[Table 11]

	b_1	b_2	b_3
Imidazole-HCl	○	○	○
$MnCl_2$	○	○	○
L-Glutamine	×	○	×
arcenate	○	○	○
Hydroxylamine	×	○	×
ADP·Na	×	○	×
酵素液	○	×	×

[0059] Notes in addition b 1, b 2, and b 3 blank A class is shown. Moreover, it is shown that O of the above-mentioned table does not add addition and x. b 1 The thing and b 2 which extracted the substrate The thing and b 3 which extracted the enzyme A substrate and an enzyme are extracted. Volume adds ultrapure water and is 500. muL It carried out. blank at the time of enzyme activity measurement finally It is the following, and made and calculated.

blank =(b 1)+(b 2)-(b 3) [0060] P450 3 A4 in the culture which used the synthetic medium for drawing 4 Installation HepG2 GS of a cell (each MSX concentration resistant strain) The result of activity (inside of a synthetic medium) is shown. Each MSX Concentration resistance P450 3 A4 Installation HepG2 GS in a cell The difference was not found out by activity. From this, it sets to each MSX concentration resistant strain, and is P450 3 A4. It is GS that there was no difference in activity. It thinks because the gene amplification in a genetic system had not happened well. It is thought that each [these] MSX concentration resistant strain acquired resistance according to devices other than gene amplification (for example, film variation etc.). A gene amplification system will be used from now on, and it is P450 3 A4. Considering raising activity, it is GS. Gene amplification systems other than a gene (CAD genetic system etc.) must be used.

[0061]

[Example 7] 9 P450 3 A4 in . blood serum Installation HepG2 P450 3 A4 in a cell In clinical application, the plasma of Buta or Homo sapiens will touch the activity measurement method 30 with a gene recombination animal cell in KIGUNASU (cell culture equipment) in time. Then, it is P450 3 A4 as a form more near application with the following approaches. Installation HepG2 It decided to evaluate the capacity to metabolize drugs (P450 3 A4 activity) of a cell by culture using not a synthetic medium but the blood serum which is a component more near plasma. Moreover, each MSX About the concentration resistant strain, activity was measured similarly.
1) 100 mm dish It is a cell 1 x10 7 An individual, seeding. The culture medium used RDF (Gln+). The amount of culture media is 10 mL. 5 The basis of %CO 2 and 37 **, and 16 Time amount culture was carried out.
2) Sample a culture medium and they are 10mL(s) about a blood serum (FBS). testosterone which is a substrate in addition Concentration is 100. mu M It added so that it might become.
3) The basis of 5 %CO 2 and 37 **, and 2 Time amount culture. This culture supernatant 2 mL It used and activity was measured.
4) After culture termination, the cell was removed by trypsinization and the amount of cell total protein was measured.
[0062] The obtained result is shown in drawing 5 . It also sets to which cell strain and is P450 3 A4. Activity is 200 pmol/min/mg-protein. The value of order was shown. This is P450 3 A4 measured in the synthetic medium. Activity is 5 about 2/. It was a value. However, MSX GS by addition P450 3 A4 of genetic system gene amplification The effectiveness in activity was not seen.
[0063]
[Example 8] 10 .P450 3 A4 Installation HepG2 Measuring method 31 former P450 3 A4 of the lidocaine metabolic turnover ability in a cell As a substrate when measuring activity, testosterone (hydroxylation like 6 beta) which is the hormone matter was used. P450 3 A4 Installation HepG2 It is testosterone when it assumes using a cell as a biotechnology artificial liver. It is necessary to evaluate also about the metabolic turnover of the drug of an except (check). Then, it decided to measure the metabolic turnover ability of the lidocaine currently used widely in case biotechnology artificial liver capacity to metabolize drugs is evaluated. Lidocaine is used as the model substrate of the first phase reaction, and a clinical index at the time of liver failure.
- HPLC Conditions HPLC Equipment -- Shimadzu LC10AD Liquid chromatograph system-usage column -- C 18 nertsil ODS-3V which are a column () [4.6 x150 mm, 5 mu m,] [GL Sciences] Inc. ** 20 mM NaClO 4 (pH 2.5) 15%acetonitrile [-- 2.0 ml/min column temperature / -- ****** / Law] style (degassing was performed) ** wavelength -- 205 nm [0064]) ** buffer -- The result is shown in Table 12. this table shows -- as -- P450 3 A4 nstallation HepG2 the lidocaine metabolic turnover ability in a cell -- 5.1 nmol/min/mg-protein it was . In addition, a value is the 2 times measurement average and each measured value is 4.7 and 5.5.
[0065]
[Table 12]

細胞株	P450 活性 (lidocaine 3- hydroxylation 活性, nmol/min/mg-protein)
P450 3A4-HepG2	5.1
HepG2	<0.1

[0066]
[Example 9] 11. It examined whether it would continue at the maintenance of drug metabolism activity over a long period of time, and also a long period, and the cell strain of this invention could maintain activity. Therefore, HepG2 incorporating CYP3 A4 and GS which are the cell strain of this invention was cultivated more than for 80 days (about 20 or more generations), and it measured about the drug metabolism activity. The obtained result is shown in the following table 13. Consequently, it became clear that the high activity of about 426 pmol/min was maintained per protein mg.
[0067]
[Table 13]

Table GS-P450 3A4 発現ベクター導入の効果

	P450 3A4 活性 (testosterone 6β-hydroxylation activity) (pmol min^{-1} $mg\text{-}protein^{-1}$)
CHO-K1 細胞(野生株)	< 0.1
P450 3A4 導入 CHO 細胞	21
HepG2 細胞 (野生株)	0.6
P450 3A4 導入 HepG2 細胞	490
P450 3A4 導入 HepG2 細胞(100%血清培養)	200
P450 3A4 導入 HepG2 細胞(80 日間以上、約 40 世代継代後)	426
ヒト初代肝細胞(生体より単離 24 時間後)	250*
ヒト初代肝細胞(生体より単離 96 時間後)	11**
ヒト生体肝	1000～1500***

*Journal of Hepatology 31:549 (1999)
**BiochemicalSocistyTransactions 22:131S (1994))
***Biochemical Pharmacology 40:2525 (1990)

[0068]
[Example 10] 12 evaluation 12.1 of the capacity to metabolize drugs as a biotechnology artificial liver based on . path clearance the path clearance theory of session -- here -- P450 3 A4 Installation HepG2 It decided to evaluate the capacity to metabolize drugs as a biotechnology artificial liver at the time of applying this cell to KIGUNASU (cell culture equipment) based on the lidocaine metabolic turnover ability of a cell. Moreover, 38, 39, and 40 it is in charge of this evaluation, and using path clearance as an index. Generally path clearance shows the magnitude of the capacity for an organ to process a drug. Processing here is a view including all, such as not only a metabolic turnover but migration which penetrates disappearance and the biomembrane of a drug. Path clearance (CL) (ml/min) is the concentration in blood (C) (mg/ml). When a drug is processed at a rate Vel (mg/min), it defines as a degree type.
Vel =CL and C -- here, it is expressed with a bottom type when the path clearance on the basis of drug concentration C in in blood (inside of plasma) (mg/ml) which flows into a biotechnology artificial liver is defined as biotechnology artificial hepatic clearance (CL artificial liver) (ml/min).
Vel =CL An artificial liver and C in [0069] 12 Plasma of Drug Concentration C in (Mg/Ml) Flows into Biotechnology Artificial Liver by the Rate of Flow Q (Ml/min) Here. Count of .2. Path Clearance -- P450 3 A4 Installation HepG2 Considering the model which drug concentration becomes small with C out (mg/ml) by the metabolic turnover by the cell, and flows out The inflow rate V in (mg/min) and exit velocity V out (mg/min) of a drug The processing speed Vel (mg/min) in a biotechnology artificial liver is an inflow rate of a drug, respectively. : Exit velocity of V in =Q and C in drug : V out =Q and processing speed in a C out biotechnology artificial liver : Vel =CL It is expressed an artificial liver and C in. It is the amount change rate of drugs in a biotechnology artificial liver (V) from this. (mg/min) is V = Vin-Vout-Vel=Q(C in-C out)-CL. An artificial liver and C in .... It is expressed **. At a steady state, since there is nothing, drug concentration change within a biotechnology artificial liver is V = 0 here. It becomes and is CL. Artificial liver = it is set to Q-(C in-C out)/C in.
Here (C in-C out) /C in=Er .... It is CL if it sets with **. Artificial liver = Q-Er .. It becomes **. Er Extraction efficiency, a call, and this express the rate that a drug is processed, while plasma passes a biotechnology artificial liver.
[0070] 12 In case it asks for the related biotechnology artificial hepatic clearance (CL artificial liver) of .3. biotechnology artificial hepatic clearance and intrinsic clearance, intrinsic clearance (CL int) must be considered. the throughput which a biotechnology artificial liver originally owns with intrinsic clearance first on the basis of the effective drug concentration (Ce) of the minute part where the drug in a biotechnology artificial liver disappears -- so to speak -- P450 3 A4 Installation HepG2 What is necessary is just to think that the throughput in a cell is expressed. As an assumption, the uncombined mold drug concentration (f and C) which has not been combined with the plasma protein in plasma etc. has the uncombined mold drug concentration of the processing part in a biotechnology artificial liver, and the relation of a concentration balance, and presupposes that only an uncombined mold drug is processed here. Here, it is f. A binding fraction with the plasma protein of a drug is expressed. if it thinks that the effective drug concentration in a biotechnology artificial liver (Ce) is uniform, and equal to the uncombined-among plasma mold concentration (f-C out) flowing out -- f and C out=Ce ** Vel =dn/dt =CL It becomes an artificial liver, C in =CL int, Ce=CL int, f, and C out ....**.

[0071] 12 Set at a ** ceremony in a .4. biotechnology artificial hepatic clearance steady state, and it is V = 0. They are [becoming and] Q(C in-C out) =CL int, f, and C out from ** type. It becomes.
From this formula and ** type, it is extraction efficiency Er. It becomes Er =f and CL int/(Q+f and CL int), and is CL from ** type. Artificial liver =Q -f and CL int/(Q+f and CL int) .... It becomes **.
[0072] 12.5 . intrinsic clearance P450 3 A4 Installation HepG2 if the drug metabolism by the cell assumes that a Michaelis-Menten equation is followed -- the substrate concentration of a metabolic turnover part, i.e., the effective drug concentration in a biotechnology artificial liver, -- Ce and Ce the maximum metabolic rate of the drug in infinity -- V max and a Michaelis constant -- K m ** -- if it carries out, it will become Vel =V max and Ce/(K m+Ce). Moreover, from being expressed with ** type, intrinsic clearance is CL int =V max/(K m+Ce).... It is expressed **.
[0073] 12 Trial calculation (cell-culture equipment: about [number of cells] 4 x109 cells and volume 1 L) 41 and 42 of .6. biotechnology artificial hepatic clearance, for example, KIGUNASU which is indicated by JP,06-113818,A, P450 3 A4 The trial calculation of biotechnology artificial hepatic clearance was made having assumed that it was filled up with introductory HepG2 cell. Already measured P450 3 A4 K m =65 in lidocaine metabolic turnover ability 5.1 nmol/min/mg-protein in introductory HepG2 cell, and a lidocaine metabolic turnover muM43 (for reference discount) And lidocaine average concentration Ce=25 in the blood in the effective lidocaine concentration in a biotechnology artificial liver, i.e., a steady state, muM43 To a radical, it is CL int from ** type. The value was computed. Consequently, CL int =57 ml/min It was able to be found. this value and flow-velocity Q=15 ml/min 41 n KIGUNASU (cell culture equipment) and -- if the trial calculation of biotechnology artificial hepatic clearance is made based on binding fraction (reference value) f =0.3 43 with the plasma protein of lidocaine -- CL Organ = 8.0 ml/min It was able to be found. This showed the value almost equivalent to the biotechnology artificial liver (CL =7.0 ml/min) 44 which used rat founder hepatocyte. Therefore, P450 3 A4 Installation HepG2 It turned out that the biotechnology artificial liver using a cell has the function of the same order as the biotechnology artificial liver using the first hepatocyte in drug metabolism. The first hepatocyte is P450 3 A4 which has reproductive integrity, when the supply from a living body is required and it takes into consideration that there is danger of a thing and virus infection with it difficult [to maintain a liver function over a long period of time] etc. Installation HepG2 Probably, a cell will be useful as a cell used for a biotechnology artificial liver. However, in computing biotechnology artificial hepatic clearance, it must care about that it is greatly dependent on the flow velocity which flows not only into the function which the cell itself with which it is filled up in cell culture equipment has but into a biotechnology artificial liver. for example, the hepatic clearance in a Homo sapiens liver -- about 700 ml/min 45 it s . the flow velocity in a Homo sapiens liver although this is about 100 times the value of the biotechnology artificial liver whose trial calculation was made previously -- about 1600 ml/min 40 it is -- about 100 of the flow velocity of a biotechnology artificial liver (KIGUNASU: 15 ml/min) It is twice.
[0074] 13. in the equipment and the culture condition of the above [actual condition / artificial liver auxiliary experiment], it is the cell strain of this invention The artificial liver auxiliary experiment was conducted using CYP3 A4-GS-HepG2. The actual liver failure model was shown in drawing 6 , and the definition of effective terminal time used for evaluation of an artificial liver auxiliary system was shown in drawing 7 . Drawing 8 shows the result of the effective terminal time in each experimental group. "None of no taking a measure" forge-fire bulk here The group which is not, the thing which connects and circulated the artificial liver system in the condition that an "acellular people liver" does not have a cell, The group for which the approach by which "plasma exchange +CHDF (continuous hemofiltration)" is generally used for the therapy of liver failure, and "GS-HepG2" used the cell strain of this invention, and "Wt-HepG2" are the groups which used the wild strain (cell strain before introducing a gene). The result shown in drawing 8 shows that the outstanding prolongation-of-life effectiveness was acquired by the liver function auxiliary device of this invention. Furthermore, as shown in drawing 9 and drawing 10 , the improvement effect which was excellent in brain pressure sthenia manifestation frequency and a list in the APTT activity partial TRON boss RASUCHIN activity in a blood coagulation system, ACT activated clotting time, HPT HEPAPURASUCHIN time amount, and various parameters called the 7th factor of FVII was accepted.
[0075]
[Example 11] Nifedipine was added into the drug metabolism assay system cell which used this invention cell strain (CYP3 A4-GS-HepG2), and the nifedipine and hydroxylation object concentration of extracellular fluid were measured with time. In 100nM(s), detection of the hydroxylation object of 5% considerable amount of an addition was attained after [of addition] 5 minutes, and it increased linearly to 50% in 30 minutes. Moreover, in 60 minutes,

t became 75%, and the parent drug became below detection sensitivity at this time. On the other hand in the :arget cell, at least 60 minutes were an average of 7%. (drawing 11) if nifedipine is added for the ketoconazole of 100microM which is 3 A4 inhibitor, cimetidine, and an erythromycin to this system after pretreatment for 10 ninutes -- the amount of hydroxylation object detection of 30 minutes after -- the time of un-processing -- it)ecame 25-35%, respectively. (Drawing 12) On the other hand, even if it used midazolam instead of nifedipine and)erformed hydroxylation object measurement, the amount of hydroxylation object detection became 35-45% at the :ime of un-adding by ketoconazole, cimetidine, and the erythromycin pretreatment. (Drawing 13) . Therefore, it is)ossible to evaluate simple whether the 3 A4 inhibition effectiveness is in that drug by adding a strange drug into :his cell and measuring hydroxylation of nifedipine and midazolam. It is the description that metabolic turnover ıbility is so high that the hydroxylation object which especially flowed out into culture medium in this cell is neasurable.

0076] bibliography 1 Watanabe Meiji .1994. clinical liver failure study . Nagai Shoten .p.p.163-s167.2 Igai ** the /olume on Kazuo .1999. liver function auxiliary device . Tokyo Women's Medical College medical engineering ·esearch facility -- the point which will clear .21 century Edge medical . Newton Press .p1463 picture Nozawa growth .1999. highly advanced medical treatment research enterprise hearing data . National Children's Hospital .4 to Hisafumi .1994. new iatrotechnique development research participation application .5 Ota Biology . University of Tokyo Press .p38 -85.6 Nagaki of Toshiji .1990. liver Trend . Japan clinical 52 of the Masahito .1994. total opinion ıybrid mold artificial liver research : 247 -252.7 Yoshida Fumitake .1993. chemical engineering, artificial organ . KYORITSU SHUPPAN .p273-274.8Rozga J, and Demetriou AA.1995.Artificial liver evolution and future)erspectives.ASAIO Journal 41:831-837.9Chen SC, Hewitt WR, and Watanabe FD, Eguchi S, Kahaku E, Middleton Y, Ronza J, and Dometriou AA.1996.Clinical experience with a porcine hepatocyte-based liver support ;ystem.International Journal of Artificial Organs 19:664-669.10Nyberg SL, Shatford RA, and Peshwa MV, White JG, Cerra FB, and Hu WS.1993.Evaluation of ahepatocyte-entrapment hollow fiber bioreacter : A potential bioartificial iver.Biotechnology and Bioengineering 41:194-203.11Nyberg SL and Misra SP.1998.Hepatocyte liver-assist ;ystems ?a clinicalupdate.Mayo Clinic Proceeding 73:765- 771.12Friedman AL.1998.Why bioartificial liver support ·emains the Holy Grail.ASAIO Journal 44:241-243.13Sussman NL and Killy JH.1993.Improved liver function ollowing treatmentwith an extracorporeal liver assistant device.Artif.Organs 17:27-30.14Enosawa S, Suzuki S, and Kkefuda T and Amemiya H.1996.Examination of 7-ethoxycoumarin deethalation and ammonia removal activities in 5:39 to 31 hepatocyte cell lines.Cell-Transplant 4015 Yamanaka Development .16 Kato of the liver origin ammonia metabolic turnover cell for a tribute .1999. Osaka University master's-thesis . fulminant hepatitis therapy Ryuichi and **** ** 41 to ** .2000. drug metabolism study . Tokyo Kagaku Dojin .p43.17 Imai Yoshiro and **** ** ** .1998.P450- the various functions and application-protein Nucleic acid Enzyme 43:203 -215.18 Nakasa **** and Kitada light 1.2000. cytochrome P-450 . monthly drug regulatory affairs the 42:746 -753.19 administration of a country ** A history .1992. hybridoma fermentation engineering-research Osaka University doctoral dissertation .20 Kobayashi of the used monoclonal antibody production the number of Hideki .1996. cells -- counting -- method . cell culture technical (edition [third] Chapter 1) . Asakura Publishing .pp. -- Pritchard 26 to 28.21 Blake J M, Ding S, Smith G, and Burchell B, Wolf C, and Friedberg.T.1996.Coexpressionof human P450(CYP3 A4) and P450 reductase generates a highlyfunctional monooxygenase system in Escherichia coli . FEBS Letters 397:210-214.22Rauschenbach R, Gieschen H, Husemann M, Salomon B, and Hildebrand M.1995.Stable expression of human cytochrome P450 3 A4 in V79 cells and its application for metabolic profiling of ergot derivatives.Euro.J.Pharm.293:183-190.23Ding S, Yao D, Burchell B, Wolf C, Friedberg.T.1997.High level s-of-·ecombinant-CYP expression-in-chinese hamster-ovary-cells are-modulated-by-coexpressed human P450 ·eductase and hemin 34.supplementation.Arch.Biochem.Biophys 348:403-410.24)Gil-L,Orellana-M,Mancilla-J,Garcia J,Vasquez H.1986.Liver microsomalepoxide hydrase.Anal.Lette 19:2261-275.25Lionel G, Miriam O, Jerson M, Juan G, and Hernan V. 1986.Use of a new HPLC method in rat liver microsomal testosterone monooxygenation and its application to study the sex dependent expressionof several hydroxylases.Analytical letters 19:2261-2275.26Williams J and Chenery R and Hawksworth G.1994.Inductionof CYP3 A enzymes inhuman and rat hepatocyte cultures.Biochem.Society.Trans 22:131. 27Dawson J and Adams D and Wolf C.1985.Induction of drug metabolizing enzymes in human liver cell line HepG2.FEBS.Lette 183:219-222.28Sumida A, Yamamoto I, Zhou Q, and Morisaki T and Azuma J.1999.Evaluation of induction of CYP3A mRNA using the HepG2 cell lineand reverse transcription-PCR.Biol.Pharm.Bull 22:61-65.29pBudCE4 Catalog no.V532-20.invitrogen.30 Naoko Tanimura .1998. Ha The ammonia metabolic turnover capacity to have been suitable for the eve lid mold artificial liver construction and functional evaluation of the cell which it has Osaka University master's thesis . 31Mario JB and Aoyama T,

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< NOTICES *

DESCRIPTION OF DRAWINGS

Brief Description of the Drawings]
Drawing 1] The result obtained in the Homo sapiens liver drug-metabolizing enzyme induction medicine rifampicin addition culture experiment to wild strain HepG2 cell is shown.
Drawing 2] P450 3 A4 A gene and GS The photograph acquired by the 0.8 % agarose gel electrophoresis of the PCR product of a gene is shown.
Drawing 3] P450 3 A4 Installation HepG2 P450 3 A4 in a cell (each MSX resistant strain of concentration) Activity (inside of a synthetic medium) is shown.
Drawing 4] P450 3 A4 GS of introductory HepG2 cell (each MSX concentration resistant strain) The result of activity (inside of a synthetic medium) is shown.
Drawing 5] P450 3 A4 in a blood serum P450 3 A4 in introductory HepG2 cell The result of activity measurement is shown.
Drawing 6] An actual liver failure model is shown.
Drawing 7] The definition of effective terminal time used for evaluation of an artificial liver auxiliary system is shown.
Drawing 8] The result of the effective terminal time in each experimental group is shown.
Drawing 9] The result of brain pressure sthenia manifestation frequency is shown.
Drawing 10] The result related with the various parameters in a blood coagulation system is shown.
Drawing 11] The situation of the metabolic turnover of the nifedipine by this invention cell strain is shown.
Drawing 12] The effect of each inhibitor to a nifedipine metabolic turnover is shown.
Drawing 13] The effect of each inhibitor to a midazolam metabolic turnover is shown.

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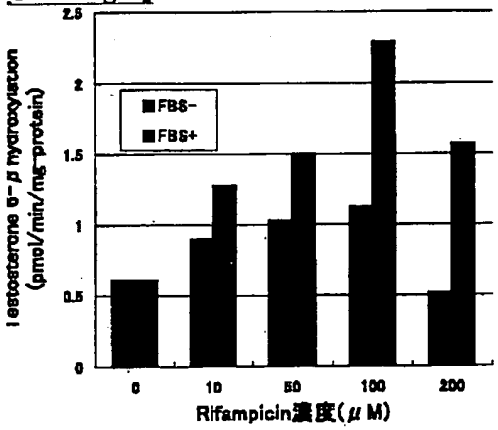
* NOTICES *

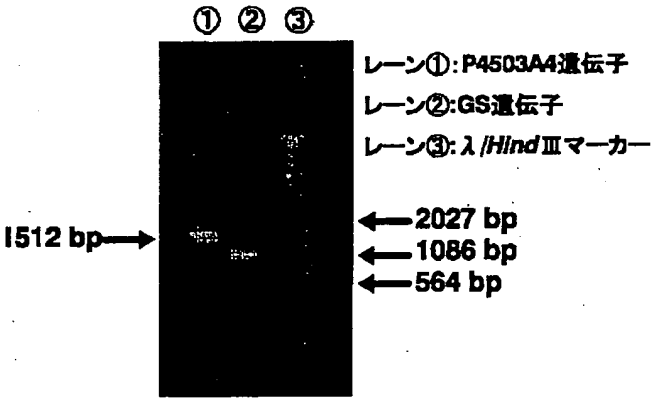
1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DRAWINGS

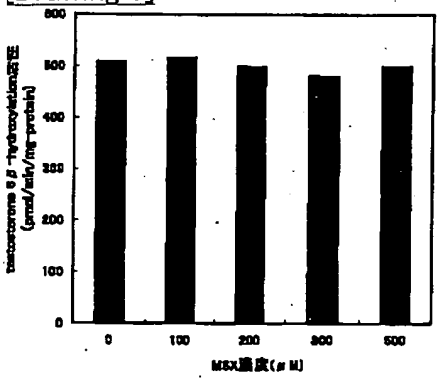
[Drawing 1]



[Drawing 2]



[Drawing 3]



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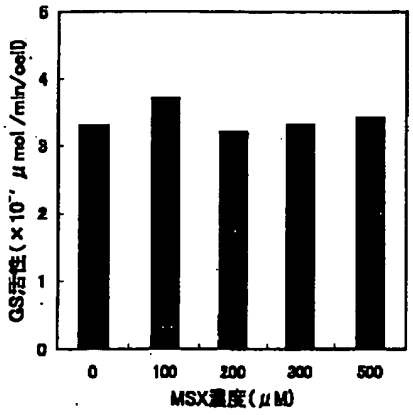

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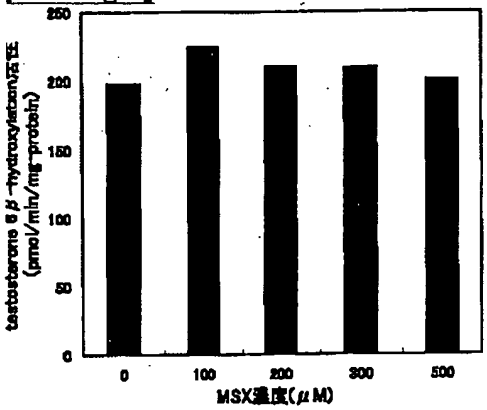
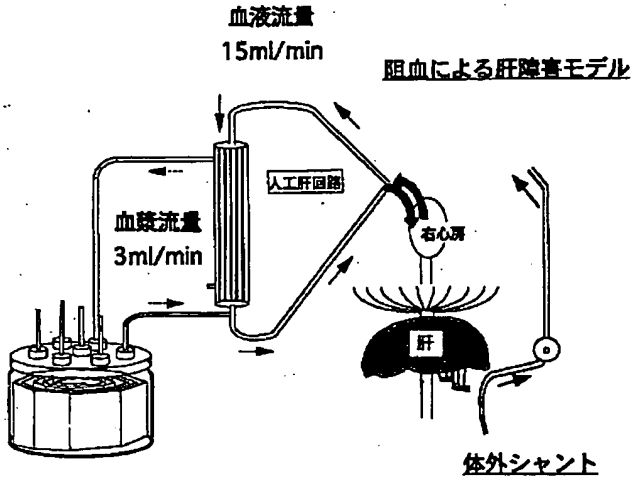


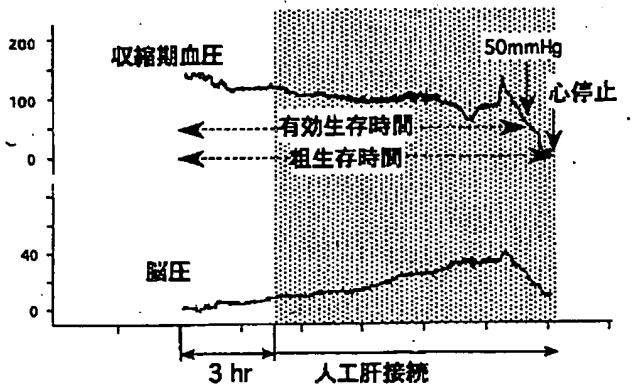
[Drawing 6]

回流式培養装置（キグナス）を用いた
ハイブリッド人工肝



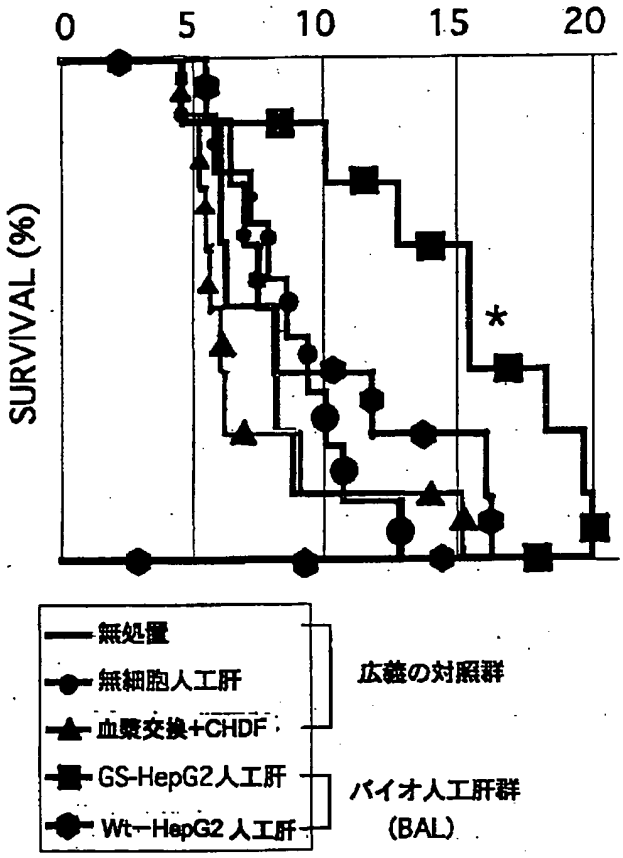
[Drawing 7]

阻血性肝不全ブタの血圧・脳圧の推移と粗生存時間、有効生存時間



[Drawing 8]

各実験群の有効生存時間の比較



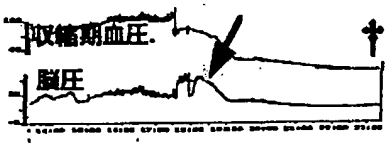
[Drawing 9]

BAL処置群と他群における脳圧亢進発現頻度の比較

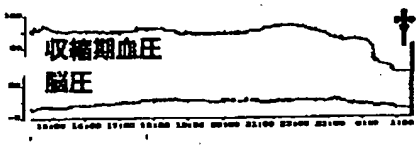
脳圧	HepG2BAL	他群
亢進例	2	11
非亢進例	14	14

カイ２乗検定, p=0.0345

1

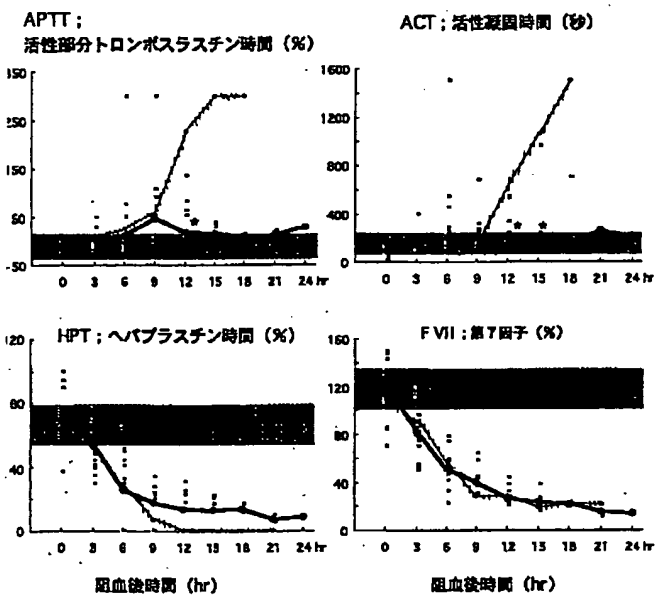


2



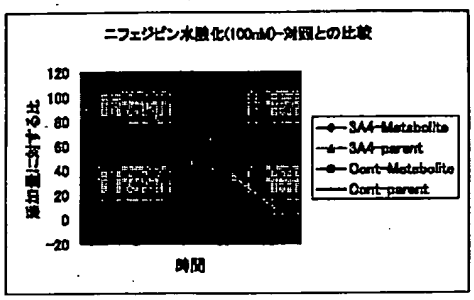
[Drawing 10]

BAL処置有(－) 無 (⁂)で比較した場合の血液凝固系の推移 （中央値）



[Drawing 11]

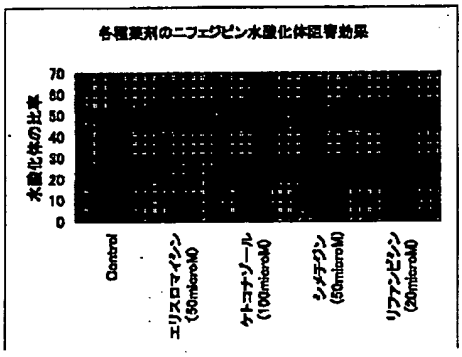
Time(min)	3A4 Metabolite	SD	Parent	SD	Control Metabolite	SD	Parent	SD
1min	0		100		0		100	
5min	9	2	93	3	0		99	1
10min	20	4	48	5	0		98	2
20min	52	5	35	6	2	2	94	4
30min	77	7	7	6	7	4	92	4



[Drawing 12]

ニフェジピン(100nM)30分添加時水酸化体産生に対する阻害薬の影響
無添加量を100%としたとき

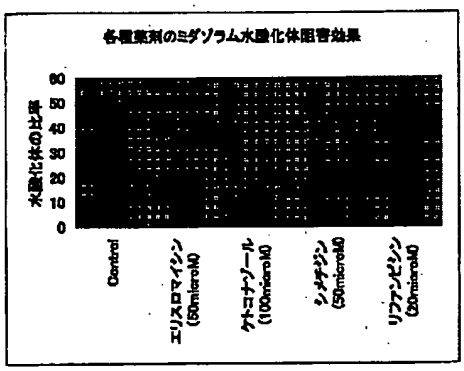
	%	SD
Control	52	5
エリスロマイシン(50n	13	4
ケトコナゾール(100m	17	4
シメチジン(50microM	19	6
リファンピシン(20mic	55	7



[Drawing 13]

ミダゾラム(250nM)30分添加時水酸化体産生に対する阻害薬の影響
無添加量を100%としたとき

	%	SD
Control	48	6
エリスロマイシン(50n	15	5
ケトコナゾール(100m	12	3
シメチジン(50microM	10	4
リファンピシン(20mic	52	5



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(19)日本国特許庁（ＪＰ）　(12)公開特許公報（Ａ）　(11)特許出願公開番号
特開2003－274963
（P2003－274963A）
(43)公開日　平成15年９月30日(2003.9.30)

(51)Int.Cl.⁷	識別記号	ＦＩ		テーマコード（参考）
Ｃ１２Ｎ　15/09	ＺＮＡ	Ａ６１Ｍ　1/36	５６９	４Ｂ０２４
Ａ６１Ｍ　1/36	５６９	Ｃ１２Ｑ　1/02		４Ｂ０６３
Ｃ１２Ｎ　5/10		Ａ６１Ｋ　35/14	Ｚ	４Ｂ０６５
Ｃ１２Ｑ　1/02		Ａ６１Ｐ　1/16		４Ｃ０７７
// Ａ６１Ｋ　35/14		7/08		４Ｃ０８７

審査請求　未請求　請求項の数27　ＯＬ　（全 22 頁）　最終頁に続く

(21)出願番号　特願2002－81344(P2002－81344)

(22)出願日　平成14年３月22日(2002.3.22)

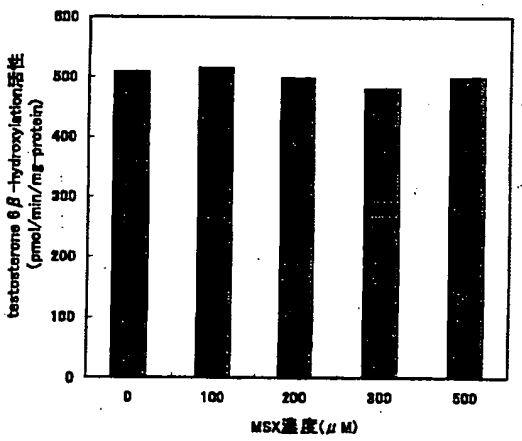
特許法第30条第１項適用申請有り　平成13年12月15日開催の「第４回移植遺伝子工学研究会」において文書をもって発表

(71)出願人　396020800
科学技術振興事業団
埼玉県川口市本町４丁目１番８号
(71)出願人　502196050
国立成育医療センター総長
東京都世田谷区大蔵２丁目10－１
(72)発明者　大政　健史
大阪府茨木市美穂ヶ丘19　D606
(72)発明者　菅　健一
大阪府箕面市粟生間谷東６－12－４
(74)代理人　100102668
弁理士　佐伯　憲生

最終頁に続く

(54)【発明の名称】　遺伝子組換え細胞株及びそれを用いた肝機能補助装置

(57)【要約】
【課題】　本発明の目的は、薬物代謝酵素遺伝子及びグルタミン合成酵素遺伝子等のアンモニア代謝酵素遺伝子によって形質転換された薬物代謝能を有する細胞株を提供し、その機能評価をし、更にこのような細胞株を利用した薬物代謝アッセイ系、及び、毒性物質の選択的除去が可能なハイブリッド型人工肝臓等の肝機能補助装置を提供することである。
【解決手段】　本発明は、Ｐ４５０　３Ａ４等の薬物代謝酵素遺伝子及びグルタミン合成酵素遺伝子等のアンモニア代謝酵素遺伝子によって形質転換された細胞株、特に、例えば、ＨｅｐＧ２細胞株のようなヒト肝臓由来である細胞株、これら細胞株を使用するハイブリッド型人工肝臓等の肝機能補助装置、及び、これら細胞株を使用する薬物代謝アッセイ系などに関する。



【特許請求の範囲】

【請求項１】　薬物代謝酵素遺伝子及びアンモニア代謝酵素遺伝子によって形質転換された細胞株。

【請求項２】　薬物代謝酵素遺伝子がＰ４５０である、請求項１記載の細胞株。

【請求項３】　薬物代謝酵素がＰ４５０　３Ａ４である、請求項２記載の細胞株。

【請求項４】　アンモニア代謝酵素遺伝子がグルタミン合成酵素遺伝子である、請求項１～３のいずれか一項記載の細胞株。

【請求項５】　細胞が哺乳類動物由来である、請求項１～４のいずれか一項記載の細胞株。

【請求項６】　細胞がヒト肝臓由来である、請求項５記載の細胞株。

【請求項７】　細胞がヒト肝実質細胞由来である、請求項６記載の細胞株。

【請求項８】　細胞がＨｅｐＧ２である、請求項７記載の細胞株。

【請求項９】　細胞が遺伝子組み換えアンモニア代謝ヒト肝細胞株である、請求項１～８のいずれか一項記載の細胞株。

【請求項１０】　薬物代謝酵素遺伝子及びアンモニア代謝酵素遺伝子が共通の発現ベクターに導入されていることを特徴とする、請求項請求項１～８のいずれか一項記載の細胞株。

【請求項１１】　発現ベクターがプラスミドである、請求項１０記載の細胞株。

【請求項１２】　発現ベクターが二つ以上の独立したマルチクローニングサイトを有する哺乳類細胞発現ベクターである、プラスミドである、請求項１０又は１１記載の細胞株。

【請求項１３】発現ベクターｐＢｕｄＣＥ４である、請求項１２記載の細胞株。

【請求項１４】　蛋白質mg当たり約２００pmol/min以上のＰ４５０活性を示すことを特徴とする、請求項１乃至１３のいずれか一項に記載の細胞株。

【請求項１５】蛋白質mg当たり約４９０pmol/min以上のＰ４５０活性を示すことを特徴とする、請求項１４に記載の細胞株。

【請求項１６】　蛋白質mg当たり約４２０pmol/minのＰ４５０活性が８０日間に亘って維持されることを特徴とする、請求項１４又は１５に記載の細胞株。

【請求項１７】請求項１乃至１６のいずれか一項に記載の細胞株を含む、肝機能補助装置。

【請求項１８】更に、別の薬物代謝酵素遺伝子で形質転換されている、請求項１乃至１７のいずれか一項に記載の細胞株。

【請求項１９】請求項１乃至１７のいずれか一項に記載の細胞株を使用する、肝機能補助装置。

【請求項２０】更に、別種の細胞株を使用する、請求項１９に記載の細胞株を含む、肝機能補助装置。

【請求項２１】別種の細胞株がヒト肝非実質細胞由来である、請求項２０記載の肝機能補助装置。

【請求項２２】ハイブリッド型人工肝臓である、請求項２０又は２１記載の肝機能補助装置。

【請求項２３】回流式培養装置を含む、請求項２０乃至２２のいずれか一項に記載の肝機能補助装置。

【請求項２４】　請求項１乃至１７のいずれか一項に記載の細胞株を使用する、薬物代謝アッセイ系。

【請求項２５】a)請求項１乃至１７のいずれか一項に記載の細胞株を培養し、
b)測定対象物質を培地に添加して更に所定期間培養し、
c)上澄みを採取し、
d)採取した上澄み中の測定対象物質及び代謝された測定対象物質の濃度をそれぞれ測定し両者の濃度比を求め、
e)測定対象物質及び適当な濃度の被験物質を培地に添加して更に所定期間培養し、
f)上記ステップc) 及び d) を繰り返し、
g)濃度比の変化から被験物質による測定対象物質の代謝に及ぼす効果を測定することから成る、
薬物代謝アッセイ系。

【請求項２６】a)請求項１乃至１７のいずれか一項に記載の細胞株を培養し、
b)ニフェジピンを培地に添加して更に所定期間培養し、
c)上澄みを採取し、
d)採取した上澄み中のニフェジピン及び酸化型ニフェジピンの濃度をそれぞれ測定し両者の濃度比を求め、
e)ニフェジピン及び適当な濃度の被験物質を培地に添加して更に所定期間培養し、
f)上記ステップc) 及び d) を繰り返し、
g)濃度比の変化から被験物質による酸化型ニフェジピン生産に及ぼす効果を測定することから成る、薬物代謝アッセイ系。

【請求項２７】a)請求項１乃至１７のいずれか一項に記載の細胞株を培養し、
b)ミダゾラムを培地に添加して更に所定期間培養し、
c)上澄みを採取し、
d)採取した上澄み中のミダゾラム及び酸化型ミダゾラムの濃度をそれぞれ測定し両者の濃度比を求め、
e)ミダゾラム及び適当な濃度の被験物質を培地に添加して更に所定期間培養し、
f)上記ステップc) 及び d) を繰り返し、
g)濃度比の変化から被験物質による酸化型ミダゾラム生産に及ぼす効果を測定することから成る、薬物代謝アッセイ系。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】本発明は、薬物代謝酵素遺伝子及びアンモニア代謝酵素遺伝子によって形質転換された細胞株、及び、該細胞株を使用する肝機能補助装置な

どに関する。

【０００２】

【従来の技術】我が国において、ウイルス性肝炎を始めとする急性肝不全患者は数多い。急性肝不全は、急激な肝細胞壊死あるいは肝細胞機能不全により、肝性脳症・黄疸・腹水・出血傾向・腎不全などを呈す。これらに対して、血液濾過透析（持続的血液濾過透析continuous arteriovenous hemofiltration ：CHDF ）や血漿交換（plasma exchange ：PE ）を組み合わせた肝機能補助療法が、現在臨床の場において実施されている。これは、肝臓により解毒・代謝されるべき物質を血液から濾過するか、または血漿と共に除去して不足分をヒト正常血漿により補充する方法である。しかし、多種にわたる肝機能の全てを代償できないため、十分な治療成績をおさめるには至っていない1) ,2) ,3) 。肝移植は急性肝不全の確立された治療法であるが、ドナー不足は極めて深刻な問題である4) 。ドナー肝が移植されるまでの緊急対応((bridge use)として、肝不全に陥った生体の肝機能を補助できる肝機能補助装置の開発は重要な課題である。また肝細胞は、非常に旺盛な再生能力を有する5) 。そのような面からも、急性肝不全に陥った肝臓が再生し機能を回復するまでの期間、肝機能補助装置を用いて肝機能を補助することの重要性は高い6) 。

【０００３】このような背景のもと、肝機能補助装置のひとつとしてハイブリッド型人工肝臓（バイオ人工肝）が挙げられる。ハイブリッド型人工肝臓とは、バイオリアクター（物理的材料）に動物細胞（生物的材料）を組み合わせたものである7) 。人工肝臓としての機能を高めるために、バイオリアクターの構造・動物細胞の充填方法・血液の循環方法等、様々な工夫がなされている8) ～14) 。

【０００４】

【発明が解決しようとする課題】ハイブリッド型人工肝臓に用いられる動物細胞は、大きく二つに分類される。１つはブタなどの異種動物由来の分離肝細胞を用いるものである。これらは高い肝機能を有しているが、細胞調達に手間・時間を要する他、肝機能を長期間維持することはできない。また異種動物であるため、免疫拒絶反応・未知のウイルス感染等の危険性がある。そこでこれらに対峙する形で用いられているものが、HepG2 を始めとするヒト由来細胞株である。肝機能は低いが、長期にわたって維持できる、細胞調達が容易であるという特性がある。そこで我々は、このヒト由来細胞株に肝機能の幾つかを付与させることを考えた。

【０００５】これまでに本発明者らはヒト肝由来細胞株HepG2 細胞に、毒性物質のひとつであるアンモニアの除去能を付与させることに成功した15) 。そこでバイオ人工肝の機能として次に重要と考えられる薬物代謝能をヒト肝由来細胞株ＨｅｐＧ２細胞に付与させることにより、毒性物質の選択的除去システムとしてのバイオ人工肝を構築したいと考えた。ゆえに薬物代謝能を有するＨｅｐＧ２細胞株の構築及びその機能評価が我々の目的である。またこれら薬物代謝能を有する動物細胞は肝不全治療目的以外にも、ヒト肝における薬物代謝研究のモデル（薬物の毒性試験や代謝経路の特定などのモデル）としての利用も考えられる。

【０００６】

【課題を解決する為の手段】ところで、薬物代謝反応は第一相反応と第二相反応に大別される16) 。第一相反応は、酸化・還元・加水分解などによって、水酸基、カルボキシル基、及びアミノ基などの極性基が生成したり、導入される反応のことをいう。これらの官能基は第二相反応に比べ比較的小さな極性基であるが、これらの極性化により薬物は一般的に作用部位に対する親和性を失う。その結果、薬理作用あるいは生理作用が低下すると共に排泄されやすい形になる。第二相反応は、第一相反応よりも大きな置換基が導入される反応である。グルクロン酸、硫酸、いくつかのアミノ酸、及びグルタチオンなどが導入される抱合反応である。水酸基、カルボキシル基、あるいはアミノ基などの官能基を予め有している化合物も少なくないが、第一相反応によってこれらの官能基が生成したり導入されてから、第二相反応を受ける化合物が多い。抱合反応の置換基は、第一相反応で導入される官能基よりも高い極性を有しているので排泄をさらに受けやすく、薬理作用あるいは生理作用を失う。

【０００７】ヒトにおける薬物代謝の約80%は肝ミクロソームに存在するチトクロムP450 が担っている。このP450は第一相反応を担っており、様々なサブファミリーから構成されている。劇症肝炎患者においては、特に第一相反応の代謝能力が低下していると言われており、その中でも特に臨床的に重要であり、成人肝において約30%の発現量を占め多種類の薬物を代謝すると言われている17)、18) のが、P450 3A4 である。そこで本発明者は、まず動物細胞における薬物代謝能を評価する上でこのP450 3A4 活性に注目することにした。またP450 3A4 の発現ベクターを構築しこれを動物細胞に導入することにより、P450 3A4 を動物細胞で発現させることを試みた。そして得られた細胞株について機能評価を行う共に、バイオ人工肝に用いる細胞としての臨床応用の可能性について検討し、本発明を完成した。

【０００８】即ち、本発明は、以下の各態様に係るものである。

１．薬物代謝酵素遺伝子及びアンモニア代謝酵素遺伝子によって形質転換された細胞株。

２．薬物代謝酵素遺伝子がＰ４５０である、上記１記載の細胞株。

３．薬物代謝酵素がＰ４５０　３Ａ４である、上記２記載の細胞株。

４．アンモニア代謝酵素遺伝子がグルタミン合成酵素遺伝子である、上記１～３のいずれか一項記載の細胞株。

５．細胞が哺乳類動物由来である、上記１～４のいずれか一項記載の細胞株。
６．細胞がヒト肝臓由来である、上記５記載の細胞株。
７．細胞がヒト肝実質細胞由来である、上記６記載の細胞株。
８．細胞がＨｅｐＧ２である、上記７記載の細胞株。
９．細胞が遺伝子組み換えアンモニア代謝ヒト肝細胞株である、上記１～８のいずれか一項記載の細胞株。
１０．薬物代謝酵素遺伝子及びアンモニア代謝酵素遺伝子が共通の発現ベクターに導入されていることを特徴とする、上記上記１～８のいずれか一項記載の細胞株。
１１．発現ベクターがプラスミドである、上記１０記載の細胞株。
１２．発現ベクターが二つ以上の独立したマルチクローニングサイトを有する哺乳類細胞発現ベクターである、プラスミドである、上記１０又は１１記載の細胞株。
１３．発現ベクターｐＢｕｄＣＥ４である、上記１２記載の細胞株。
１４．蛋白質mg当たり約２００pmol/min以上のＰ４５０活性を示すことを特徴とする、上記１乃至１３のいずれか一項に記載の細胞株。
１５．蛋白質mg当たり約４９０pmol/min以上のＰ４５０活性を示すことを特徴とする、上記１４に記載の細胞株。
１６．蛋白質mg当たり約４２０pmol/minのＰ４５０活性が８０日間に亘って維持されることを特徴とする、上記１４又は１５に記載の細胞株。
１７．上記１乃至１６のいずれか一項に記載の細胞株を含む、肝機能補助装置。
１８．更に、別の薬物代謝酵素遺伝子で形質転換されている、上記１乃至１７のいずれか一項に記載の細胞株。
１９．上記１乃至１７のいずれか一項に記載の細胞株を使用する、肝機能補助装置。
２０．更に、別種の細胞株を使用する、上記１９に記載の細胞株を含む、肝機能補助装置。
２１．別種の細胞株がヒト肝非実質細胞由来である、上記２０記載の肝機能補助装置。
２２．ハイブリッド型人工肝臓である、上記２０又は２１記載の肝機能補助装置。
２３．回流式培養装置を含む、上記２０乃至２２のいずれか一項に記載の肝機能補助装置。
２４．上記１乃至１７のいずれか一項に記載の細胞株を使用する、薬物代謝アッセイ系。
２５．
a)上記１乃至１７のいずれか一項に記載の細胞株を培養し、
b)測定対象物質を培地に添加して更に所定期間培養し、
c)上澄みを採取し、
d)採取した上澄み中の測定対象物質及び代謝された測定対象物質の濃度をそれぞれ測定し両者の濃度比を求め、

e)測定対象物質及び適当な濃度の被験物質を培地に添加して更に所定期間培養し、
f)上記ステップc) 及び d) を繰り返し、
g)濃度比の変化から被験物質による測定対象物質の代謝に及ぼす効果を測定することから成る、薬物代謝アッセイ系。
２６．
a)上記１乃至１７のいずれか一項に記載の細胞株を培養し、
b)ニフェジピンを培地に添加して更に所定期間培養し、
c)上澄みを採取し、
d)採取した上澄み中のニフェジピン及び酸化型ニフェジピンの濃度をそれぞれ測定し両者の濃度比を求め、
e)ニフェジピン及び適当な濃度の被験物質を培地に添加して更に所定期間培養し、
f)上記ステップc) 及び d) を繰り返し、
a)濃度比の変化から被験物質による酸化型ニフェジピン生産に及ぼす効果を測定することから成る、薬物代謝アッセイ系。
２７．
a)上記１乃至１７のいずれか一項に記載の細胞株を培養し、
b)ミダゾラムを培地に添加して更に所定期間培養し、
c)上澄みを採取し、
d)採取した上澄み中のミダゾラム及び酸化型ミダゾラムの濃度をそれぞれ測定し両者の濃度比を求め、
e)ミダゾラム及び適当な濃度の被験物質を培地に添加して更に所定期間培養し、f)上記ステップc) 及び d) を繰り返し、
b)濃度比の変化から被験物質による酸化型ミダゾラム生産に及ぼす効果を測定することから成る、薬物代謝アッセイ系。
【０００９】薬物代謝酵素としては、当業者に公知の任意のもの、例えば、チトクロムＰ４５０に属する各種酵素、具体的には、Ｐ４５０　３Ａ４（ＣＹＰ３Ａ４）、ＣＹＰ２Ｃ、ＣＹＰ１Ａ２、ＣＹＰ２Ｅ１、ＣＹＰ２Ｄ６、及びＣＹＰ２Ａ６等から選択した１種又は２種以上を使用することが出来る。これらの中で、P450 3A4 は成人肝において、約30 %の発現量を占め臨床で使用される医薬品の約半分を代謝することができる。このP450 3A4 の次に多くの医薬品を代謝するP450 2D6 やP450 2C を発現させることも重要であると考えられる。特にP450 2D6 に関しては、遺伝的多型という面からも注目されている。このP450 2D6 の活性を持たない患者（PM ）は、日本人では0.8 %程度と少ないが、白人では5 ～6 %と言われており、急性肝不全患者の治療に使用するバイオ人工肝の開発という発想のみならず、このようなPMの治療に使用するバイオ人工肝の開発という観点からも重要である。アンモニア代謝酵素遺伝子の例としては、例えば、ＣＨＯ細胞由来のグルタミン合成酵素遺伝子を

挙げることができる。これらの各遺伝子は、いずれも市販、又は公的機関所有のｃＤＮＡライブラリー、又はベクター等からＰＣＲ等のクローニング手段によって、当業者であれば、容易に入手又は調製することができる。又、これら遺伝子の塩基配列は各種文献に開示されている。形質転換の対象となる細胞株は哺乳類動物由来であることが好ましく、例えば、ヒト肝臓由来の細胞株がより好ましく、かかる細胞株の一例として、ＨｅｐＧ２細胞株を挙げることが出来る。このような細胞は各種公的機関（細胞バンク）から入手することが可能である。更に、薬物代謝活性を高める等の目的で、形質転換して得られた本発明の細胞株をクローニングすることもでき、こうしてクローニングして得られた細胞株も本発明の範囲である。薬物代謝酵素遺伝子及びアンモニア代謝酵素遺伝子は、それぞれ、別の発現ベクターに担持され別個に形質転換が行われてもよいが、共通の発現ベクターに担持されていると効率的であり便利である。かかる発現ベクターとしては当業者に公知の任意のベクターを使用することが出来るが、二つ以上の独立したマルチクローニングサイトを有する哺乳類細胞発現ベクター、例えば、発現ベクターｐＢｕｄＣＥ４が好適である。尚、発現ベクターへの各遺伝子の導入、及び該発現ベクターによる細胞株の形質転換等の各操作は当該技術分野で公知の任意の方法・手段で容易に行うことが出来る。本発明の細胞株は更に、別の種類の薬物代謝酵素遺伝子で形質転換されていてもよい。このような薬物代謝酵素の例として、P450 による第一相反応のみならず第二相反応を担う薬物代謝酵素を挙げることが出来る。例えば、グルクロン酸抱合は、UDP－GT(UDP－glucuronyltransferase)によって行われるので、このUDP－GT 発現ベクターを更に導入することは有効であると考えられる。本発明の肝機能補助装置は当業者に公知の任意の構成をとることが出来るが、回流式培養装置を含むハイブリッド型人工肝臓のタイプが好適である。かかる装置において、本発明の細胞株を生物的材料として使用する訳であるが、その他の種類の細胞を更に生物的材料として使用することも出来る。本発明の薬物代謝アッセイ系は、上記細胞株を使用することを特徴とし、その細胞株による、例えば、ニフェジピン及びミダゾラム等の測定対象物質（標準物質）の代謝反応（例えば、酸化反応、水酸化反応）に及ぼす被験物質の影響を測定することによって行うことが出来る。

【００１０】以下、実施例に則して本発明を詳述するが、本発明の技術的範囲はこれらに何等拘されるものではない。

【００１１】

【実施例１】実験材料及び方法

試薬は特筆しない限り和光純薬又はナカライテスクの特級試薬を使用した。１．動物細胞培養方法

１．１．　宿主動物細胞

HepG2 (理化学研究所細胞バンクRCB0459)

由来 human hepatocellular carcinoma

増殖形態epithelial－like

【００１２】１．２．動物細胞培養培地

培地は、0.22 μM のメンブレンフィルター(Falcon ;7105)を用いて濾過滅菌した後、使用した。・RDF (Gln＋)

RDF (Gln＋)培地組成

RDF (HO)粉末（日本製薬）8.44 g

グルコース 2.58 g

NaHCO 3 2.0 g

グルタミン 0.333 g

ペニシリンG 58.8 mg

ストレプトマイシン 120 mg

Milli－Q 水 1 L

注1)使用する際には、牛胎児血清(fetal bovine serum (FBS) ;Gibco)を培地容量の10%(vol.%)相当量加えた。

注2 ）必要に応じて、Zeocin （Invitrogen ;R250－01)を加えた。・RDF (Gln－)

RDF (Gln－)培地組成

RDF (HO)粉末（日本製薬）8.44 g

グルコース 2.58 g

NaHCO 3 2.0 g

グルタミン酸 0.336 g

NH 4 Cl 0.122 g

ペニシリンG 58.8 mg

ストレプトマイシン 120 mg

Milli－Q 水 1 L

注1)グルタミン酸とNH 4 Cl の組成は、RDF (Gln+)培地のグルタミンと同じモル数になるように決定した。

注2)RDF (HO)培地は、日本製薬の特注品であり、通常のRDF 培地よりグルコース、グルタミンを抜いたものである。

注3)使用する際には、あらかじめ透析したFBS を培地容量の10%(vol.%)相当量加えた。

注4)培地にMSX を必要とする場合 ((高MSX 耐性株の取得実験に使用)は、各MSX濃度に応じてMSX (Sigma ;M－5379)を添加した。

注5)必要に応じて、使用時に geneticin ((G418)(Sigma ;G5013)及びZeocin を添加した。

・血清培養用培地

血清培養用培地組成

NaHCO 3 0.2 g

ペニシリンG (1700 units/mg)5.88 mg

ストレプトマイシン 12 mg

FBS 100 mL

透析血清

透析用セルローズチューブ(三光純薬 ; ;Size 27/32)を2%(w/v)NaHCO 3 、 1 mMEDTA (pH 8.0)に浸して10 分間オートクレーブをした後、4 ℃に冷やし、これに血清を入れ、血清の10 倍量の透析液を用いて、30 分間→1 時

間→2 時間→3 時間→4 時間→over night で透析を行った。

血清透析液

NaCl 8.0 g
KCl 0.2 g
Na 2 HPO 4 ・12H 2 O 2.9 g
KH 2 PO 4 0.2 g
カナマイシン 32 mg
ストレプトマイシン 120 mg
蒸留水 1 L

【００１３】１．１．３．動物細胞培養における基本的事項及び操作

１．１．３．１．細胞培養容器

細胞培養容器としては、3 種類のT－フラスコ ((住友ベークライト;MS－20050(底面積25 cm 2 ,容量50mL),MS－21250 (75 cm 2 ,250 mL),MS－20800 (225 cm2 ,800 mL)(以下、小T－フラスコ、中T－フラスコ、大T－フラスコと省略))または、100 mm dish (Corning ;25020)を用いた。ここで、小T－フラスコと100 mm dish には、通常10 mL の培地を、中T－フラスコには30 mL の培地を、大T－フラスコには90 mL の培地を入れた。通常の細胞株の継代培養には小T－フラスコを用いた。また培養実験では100 mm dish を用いた。

【００１４】１．３．２．細胞の継代

原則として、1 日おきに ((細胞がコンフルエント状態であることを確認して)行った。

1)古い培地をピペットで吸引して除き、以下の容量の0.25%トリプシン溶液を加えた。

小 T－フラスコ…5 mL,中T－フラスコ…15 mL,大T－フラスコ…30 mL

2)37 ℃で約10 分間培養した。

3)細胞がはがれ丸くなったら、新しい培地を当量加えてよくピペッティングした(通常血清中にはトリプシンインヒビターが入っているので、細胞をはがす反応がこの時点で止る)。

4)細胞浮遊液を遠心管に移し、80 ×g.で10 分間遠心分離した後、上澄みをピペットで吸引除去した。

5)遠心管内に新鮮培地を加えて細胞を懸濁し、新しいT－フラスコに適当量接種した。

【００１５】0.25%トリプシン溶液

NaCl 8 g
KCl 0.2 g
Na 2 PO 4 ・12H 2 O 2.9 g
KH 2 PO 4 0.2 g
TRYPSIN (Difco 1:250)2.5 g
Milli－Q 水 1 L

【００１６】１．３．３．細胞の凍結保存法

細胞の凍結保存においては、培地に10%のdimethyl sulfoxide (DMSO)を加えた液に細胞を懸濁し、セラムチューブに1 mL ずつ分注して、BICELL (Nihon Freezer)に入れ、－80 ℃で一晩凍結させた後、液体窒素中にて保存した。解凍は、37℃の温水中にて行い培地を溶かした後、10 mL の培地を入れた遠心管に細胞懸濁液を移し、80 ×g.で10 分間遠心分離した後、上澄みをピペットで吸引除去し細胞を新しいT－フラスコに接種した。

【００１７】１．３．４．細胞濃度測定方法（色素排除法による計測）19)

生細胞および全細胞濃度はトリパンブルーを用いた色素排除法を用いて測定した．これはトリパンブルー水溶液0.2%(w/v)とNaCl 水溶液4.25%(w/v)を4:1 の割合で混合し、この液に細胞浮遊液を等量混合し、Burker－Turk 型血球計算板 ((ERMA 4296)に一滴 ((約15 μ L)のせ、ただちに検鏡して生細胞濃度、全細胞濃度を計測する方法である．血球計算板では右側と下側の二辺にかかる細胞は計測時に省いた。ここで、血球計算板付属の検定値に従って血球計算板の深さの補正を行い、血球計算板上の体積を計算し、細胞濃度を算出した20)。

【００１８】２．HPLC を用いた薬物代謝活性（P450 3A4 活性）測定系

２．１．HPLC を用いた基質・代謝物の濃度測定方法21)～24)

薬物代謝活性（P450 3A4 活性）は、P450 3A4 のみによって特異的に代謝されるホルモン物質testosterone (第一化学薬品;UC－339、分子量 ； 288.4)を基質とし、代謝物である6 β－hydroxytestosterone (第一化学薬品 ;;UC－282、分子量 ； 304.4)をHPLC で測定することにより評価した。HPLC を行う際の定量方法として内部標準法を用いた。ここで内部標準物質として、androstenedione(4－Androstene－3,17－dione) (第一化学薬品 ;;UC－300)を用いた。始めに標準濃度として、基質・代謝物・内部標準物質それぞれ100μM になるように調製し、HPLC チャートのピーク面積を基に以降基質並びに代謝物の濃度を算出した。

【００１９】・HPLC 条件

HPLC 装置… 島津製作所液体クロマトグラフシステム ;LC10AD;
使用カラム …C 18 カラムであるInertsil ODS－3V (4.6 ×150 mm,5 μ m,GL Sciences Inc.);
使 用buffer … A 液）メタノール：蒸留水=45 ：55 B 液）メタノール：蒸留水＝90 ：10。

Buffer 作製においては、HPLC 用メタノール（ナカライテスク）及び蒸留水を各々の比率で混合した後、適宜脱気を行った。

流 速… 1.0 ml/min;
カラム温度 …室温;
測 定波長 … 254 nm;
P450 3A4 活性におけるHPLC gradient 条件

【００２０】

【表１】

時間(分)	A液	B液
0	100%	0%
↓	↓	↓
10	0%	100%
～12	0%	100%
12～	100%	0%

【００２１】２．２．細胞総蛋白量の定量
P450 3A4 活性は、一般的にpmol 6 β－hydroxytestosterone formed/min/mgprotein という単位で表現される。細胞培養上清を用いて薬物代謝活性を求める際には、細胞総蛋白量(mg protein)を測定しなければならない。ここでは細胞総蛋白量をBCA Protein Assay Reagent Kit (Pierce ;23225)を用いて測定することとした。定量方法は、付属マニュアルに従った。細胞総蛋白量測定における、細胞破砕液（粗酵素液）の調整手順を以下に示す。以下に手順を示す。
1)細胞を既知量の培地（100 mm dish 培養の際は、培地5 ml が目安）に懸濁し、そのうちの500 μl を遠心チューブに移し、1000 ×g で10 分間、4 ℃で遠心分離した。
2)上清を除き、100 mM potassium phosphate buffer (pH7.4)に懸濁し、1000×gで10 分間、4 ℃で遠心分離した。
3)超音波菌体破壊装置（海上電機 T－－ A－4200 ）により、5 秒間超音波処理後、1 分間氷冷し、これを4 回繰り返した。
4)14000 ×g で10 分間、4 ℃で遠心分離後、上清を用いて細胞総蛋白量を定量した。
【００２２】２．３．細胞培養上清（サンプル）の前処理25)
HPLC を用いて細胞培養上清中の基質及び代謝物濃度を測定するにあたり、サンプル前処理用カラムSep－Pak Plus C 18 （Waters ） を用いて、サンプルの精製を行った。手順を以下に示す。カラムに注入 (apply) する際の流速は、2～4 ml/min となるように留意した。
1 ）100%メタノール2 ml をカラムに2 回apply した。
2 ）蒸留水2 ml をカラムに2 回apply した。
3 ）サンプルを2 ml 、apply した。
4 ）蒸留水2 ml をカラムにapply し、カラムを洗浄した。
5 ）100%メタノール2 ml apply し、目的物質を溶出した。
【００２３】３．動物細胞（intact cell ）におけるP450 3A4 活性測定方法21) ,23)
1)10 mL の培地RDF (Gln + もしくはGln －)を用い、100 mm dish に細胞を1 ×10 7 個、播種した。培養は、5 %CO 2 、37 ℃のもと、16 時間行った。
2)培地交換を行い（培地量10 mL ）、基質であるtestosterone （100mM になるように100%メタノールに溶かした）を濃度が100 μ M になるように加えた。
3)5 %CO 2 、37 ℃で、2 時間培養した後、この培養上清2 mL を用いて、活性を測定した。
4)培養終了後、トリプシン処理により細胞を剥がし細胞総蛋白量を測定した。
【００２４】
【実施例２】４．内在P450 3A4 活性向上のためのヒト肝薬物代謝酵素誘導薬rifampicin 添加培養
動物細胞をバイオ人工肝に適用するにあたり、野生株HepG2 細胞及び、本発明者のよりこれまでに構築されている遺伝子組換えアンモニア代謝HepG2 細胞（GS－HepG2 細胞、MSX300 μM 耐性株）における内在性のP450 3A4 の活性の向上を試みた。これまでに、HepG2 細胞においてはdexamethasone , phenobarbital ,3－methylcholanthrene ,prednisolone ,carbamazepine ,rifampicin 等の薬剤を培地に添加すると、薬物誘導により薬物代謝能が上昇することが報告されている26) ,27) ,28) 。そこでこれら薬物誘導剤の中で、CYP3A の誘導剤として最も効果的であるとされるrifampicin を添加することにより、内在性P4503A4 活性の向上を目指した。
1)100 mm dish に、RDF 培地(10%FBS を含む)を最終的に10 mL になるように加えた。
2)野生株HepG2 並びに遺伝子組み換えGS－HepG2 細胞（HNAA－300A 細胞株）を、1 ×10 7 cells 播種した。
3)37 ℃、5%CO 2 のもと12 時間、培養した。
4)培地交換。10%FBS を含むもしくは無血清のRDF 培地を10 mL 加えた。
5)37 ℃、5%CO 2 のもと24 時間、培養した。
6)培地交換。dimethylsulfoxide (DMSO)に溶かしたrifampicin を、最終濃度が10 ,50 ,100 ,200 ,300 ,500 μ Mになるように無血清のRDF 培地に添加。コントロールとして無血清RDF 培地にDMSO のみ(0.1%)を加えた。
7)37 ℃、5% CO2 のもと24 時間培養した後、培地交換及び各濃度のrifampicinを添加した。
8)7)を６日間行い（6 日間毎日培地交換を行った）、その後200 μ M のtestosterone を培地に添加し、P450 3A4 活性を測定した。
【００２５】上記の野生株HepG2 細胞に対するヒト肝薬物代謝酵素誘導薬rifampicin 添加培養実験で得られた結果を、図１に示した。これは培地中にFBS を含む場合と含まない場合、及びrifampicin 各濃度における野生株HepG2 細胞のP450 3A4 活性を示したものである。野生株HepG2 細胞のP450 3A4 活性は、薬物誘導をかけない状態で0.6pmol/min/mg－protein という値を示した。また最もP450 3A4 活性が高かったのは、FBS を含む培

地においてrifampicin 100 μM で誘導をかけたもので、活性値は2.3 pmol/min/mg－protein となった。培地中にFBS を含む場合と含まない場合を比較することによって、FBS を含む培養において内在性P450 3A4 が誘導されやすいことがわかった。FBS （血清）中には、成長因子を始めとする様々な重要な因子が含まれている。従ってFBS なしの培養では生存していくための重要な因子が枯渇しているため、細胞における外界の変化に対する応答が鈍くなるためではないかと考えた。

【００２６】更に、遺伝子組換えアンモニア代謝HepG2 細胞（GS－HepG2 細胞、MSX300 μM耐性株）対するヒト肝薬物代謝酵素誘導薬rifampicin 添加培養実験で得られた結果を、図２に示した。これは透析血清を含む場合と含まない場合、及び各rifampicin 濃度におけるHNAA－300A 細胞株のP450 3A4 活性である。この図からわかるように薬物誘導をかけない状態では、HNAA－300A 細胞株は、野生株HepG2細胞とほぼ同じ活性を有していた。ただしrifampicin 添加による薬物誘導では、野生株HepG2 細胞と比較してP450 3A4 の誘導はされにくいことが確認された。HNAA－300A 細胞株は、野生株HepG2 細胞にpBK－CMV－GS ベクターを導入した後、MSX 及びG418 を用いて選択をかけたものである。このような過酷な環境下でも生存できることを含めて考えると、HNAA－300A 細胞株の外界に対する感受性が鈍くなっているため、野生株HepG2 細胞に比べ、rifampicin によるP450 3A4 の誘導が起こりにくいのではないかと考えた。

【００２７】

【実施例３】５．P450 3A4 発現ベクターの構築

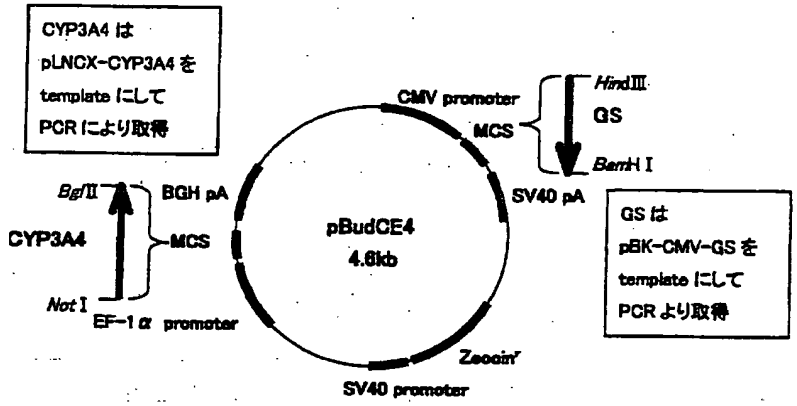
５．１．P450 3A4 発現ベクター（pBudCE－GS－CYP3A4）構築の概略

５．１．１．使用プラスミド

使用プラスミドとして、pBudCE4 （Invitrogen ;V532－20 ）29）を用いた。これは、multicloning site （MCS ）を2 ヶ所有しており、それぞれのMCS の上流にhuman cytomegalovirus (CMV) immediate－early promoter 及びhuman elongation 1 α－subsuit (EF－1 α)promoter を有する。マーカー遺伝子として、大腸菌及び動物細胞ではZeocin で選択可能である。P450 3A4 発現ベクターの構築の概略を以下の表２に示す。

【００２８】

【表２】



【００２９】５．１．２．使用プラスミドの調製

５．１．２．１．使用菌株

大腸菌TOP10 （invitrogen ;C615－00）

【００３０】５．１．２．２．培地（LB－Zeocin ）

脱イオン水100 mL に、Bacto－tryptone (Difco)1 g 、Bacto yeast extract (Difco)0.5 g 、NaCl 0.5 g を溶かした後、pH 7.0 に調製し、オートクレーブした。プレートにする場合には2 g のAgar を加えた。オートクレーブ後、55 ℃ぐらいになったら、Zeocin を50 μg/mL になるように加えた。

【００３１】５．１．２．３．塩化カルシウム法による大腸菌の形質転換

・コンピテントセルの作成

１）形質転換する大腸菌を5 mL のLB 培地で一晩37 ℃で振とう培養した。

２）前培養液2 mL を40 mL のLB 培地に植菌し、37 ℃で2 時間培養した。

３）氷中で10 分以上放置し、4 ℃、6000 ×g 、5 分間遠心分離することにより集菌した。

４）沈澱を氷冷した50 mM CaCl 2 20 mL に懸濁し、0 ℃で20 分間放置した。

５）遠心分離により集菌後、50 mM CaCl 2 4 mL に懸濁し、200 μL ずつ分注した。

・形質転換

200 μL のコンピテントセルに対して、DNA 溶液を適当量（0.01 μg 程度）加え、氷中で1 時間放置した。42 ℃で、90 秒間熱ショックを与えた後、氷水で急冷し、0.8 mL のLB 培地を加え、37 ℃で1 時間振とう培養した。この培養液のうち、100 μL をLB－Zeocin 培地に広げ、37 ℃で一晩培養した。

【００３２】５．１．２．４．大腸菌からのプラスミドDNA の調製（アルカリ抽出法）

5 mL のLB－Ampicilin 液体培地に大腸菌形質転換体を植菌し、37 ℃で16 時間振とう培養した。培養液を1.5 mL 程度取り、12000 ×g 、2 分間遠心分離し、集菌した。沈澱をボルテックスミキサーで撹拌した後、soluti

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on Ｉ100 μLに完全に懸濁し、solution ＩＩ200 μL を加え、穏やかに3 ～4 回転倒混和し氷中で正確に5 分間放置した。予冷しておいたsolution ＩＩＩを150 μL 加え、激しく混合し、氷中で5 分間放置した。12000 ×g 、5 分間遠心分離した後、上清を新しいサンプルチューブに移した。この上清について、フェノール－クロロホルム抽出を行い、さらにエタノール沈殿を行った後、20 μg/mL のDnase free RNase を含むTE 50 μL に溶解した。

solution Ｉ： 50 mM Glucose 、 25 mM Tris－－Cl(pH 8.0)、 10 mM EDTA

solution ＩＩ： 0.2 N.NaOH 、 1%%SDS （要時調製）

solution ＩＩＩ： 5 M 酢酸カリウムを含む酢酸水溶液

【００３３】５．２．遺伝子関連基本的操作

５．２．１．アガロース電気泳動

1/2 ×TAE 緩衝液を用いた0.8%アガロースゲル（TaKaRa ;Agarose LO3 ）で行った。泳動槽として、小型電気泳動槽（Advance Co.Ltd.;Mupid2 ）を用いた。試料は通常、DNA 溶液に1/10 量の×10 泳動用染色液を加え作製した。泳動は定電圧100 V で40 分程行った。泳動後、ゲルをethidium bromide (EtBr)水溶液（0.5 μ g/ml ）で10 分間染色し、トランスイルミネーター（Ultra Violet C62 ）でDNA バンドを観察した。写真は、UV フィルター及び赤色フィルターを用い、ポラロイド（登録商標）カメラで撮影した。

【００３４】５．２．２．エタノール沈殿

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DNA を含む溶液に、1/10 量の3 M 酢酸ナトリウム溶液（pH 5.2 ）を加え、さらにその溶液の2.5 倍量の100 %エタノールを加えて混合し、室温で20 分放置した。これを18000 ×g 、10 分間遠心分離し、上清を取り除いた。この沈殿に70%エタノールを適量加え、18000 ×g 、10 分間遠心分離した後、上清を取り除き沈殿を減圧乾固後、適量の滅菌水もしくは滅菌TE 緩衝液（10 mM Tris－ Cl (pH 7.5) ,1 mMEDTA ）に溶解した。

【００３５】５．２．３．フェノール－クロロホルム抽出

DNA 溶液をTE 緩衝液で飽和し、フェノール－クロロホルム－イソアミルアルコール（25:24:1 ）混合液を等量加えて良く混合した。これを18000 ×g 、10分間遠心分離し、上層を新しいサンプルチューブに移した。

【００３６】５．３． PCR によるP450 3A4 遺伝子及びGS 遺伝子の調製

・使用機器

GeneAmpR PCR System 2400 (PERKIN ELMER)

・template

P450 3A4 遺伝子については、pLNCX－CYP3A4 （Els M.De Grone 先生より寄贈）、GS 遺伝子については、PBK－CMV－GS を用いた。使用したprimerを以下の表３に示す。

【００３７】

【表３】

GS遺伝子をPCRにより獲得する際に用いたプライマー

N末端側 *Hind*Ⅲ
5' - AAAAAA**AAGCTT**ACCATGGCCACCTCAGCAAGTTCCC - 3'

C末端側 *Bam*HⅠ
5' - CCCCCC**GGATCC**AATTAGTTTTTGTATTGGAAGGGCTC - 3'

CYP3A4をPCRにより獲得する際に用いたプライマー

N末端側 *Not*Ⅰ
5' - TTTTTT**GCGGCCGC**GTGATGGCTCTCATCCCAGACTTGG - 3'

C末端側 *Bgl*Ⅱ
5' - GGGGGG**AGATCT**ATTCAGGCTCCACTTACGGTGCCATC - 3'

【００３８】なおプライマーの設計にあたり、動物細胞における翻訳がより正しく開始されるように、Kozak の配列（A/G NN ATG G ）を参考にした。また、以下の表４に、それぞれのPCR 反応の組成及び条件を示した。

【００３９】

【表４】

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primer GS *Hind*Ⅲ (20 pmol/μl)	1 μl
primer GS *Bam*H Ⅰ (20 pmol/μl)	1 μl
Template (pBK-CMV-GS) (50 pmol/μl)	1 μl
KOD polymerase buffer (×10)	5 μl
dNTP 2 mM	5 μl
$MgCl_2$ 25 mM	2 μl
KOD polymerase 2.5 unit/μl	1 μl
滅菌水	34 μl
total	50 μl

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primer CYP3A4 *Not* Ⅰ (20 pmol/μl)	1 μl
primer CYP3A4 *Bgl*Ⅱ (20 pmol/μl)	1 μl
Template (pLNCX-CYP3A4) (50 pmol/μl)	1 μl
KOD polymerase buffer (×10)	5 μl
dNTP 2 mM	5 μl
$MgCl_2$ 25 mM	2 μl
KOD polymerase 2.5 unit/μl	1 μl
滅菌水	34 μl
total	50 μl

・PCR条件

98℃ 15 sec → 65℃ 2 sec → 74℃ 30 sec

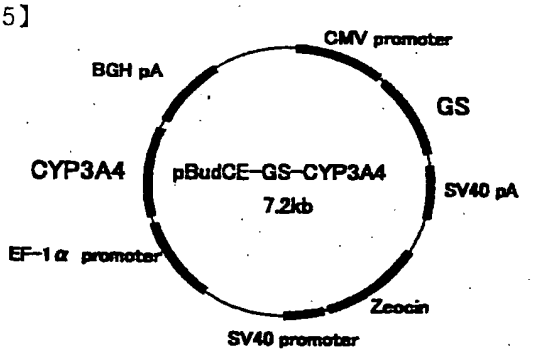
← 25 cycles →

【００４０】５．４． pBudCE4 ベクター(invitrogen)とインサートであるP450 3A4 遺伝子及びGS 遺伝子とのligation

５．１．２．４．で調製したpBudCE4 ベクター及び５．３．で調製したP450 3A4遺伝子及びGS 遺伝子を用いてライゲーションさせることにした。この過程は、宝酒造株式会社 遺伝子解析センターに外注した。この結果、得られたP450 3A4 発現ベクター (pBudCE－GS－CYP3A4) を以下の表５に示す。このベクターは、pBudCE4 ベクターの二ヶ所のmulticloning siteに、pLNCX－CYP3A4 由来のCYP3A4(P450 3A4 の遺伝子)及びpBK－CMV－GS 由来のGS 遺伝子を挿入したものである 。

【００４１】

【表５】



【００４２】更に、５．３．に従いP450 3A4 遺伝子及びGS 遺伝子をPCR により増幅し、PCR 産物4 μL を0.8 %アガロースゲルを用いて電気泳動した。その結果を図２に示す。レーン①はP450 3A4 遺伝子（約1.5kbp ）、レーン②はGS 遺伝子(約1.1kbp)、レーン③はλ/ HindⅢマーカーである。これより目的の遺伝子であるP450 3A4 遺伝子及びGS 遺伝子の獲得を確認した。P450 3A4 発現ベクターに挿入されたP450 3A4 の遺伝子(CYP 3A4)のシークエンス結果を以下の表６に示す。デ ータベースに登録されているCYP3A4 の塩基配列33) と比較すると、開始コドン（ATG ）の後の6 塩基目がC(シトシン)ではなくA(アデニン)であった。しかし蛋白に翻訳させる際にはどちら（CTC 及びCTA）とも構成アミノ酸はロイシンとなることより、ベクターに挿入したP450 3A4 は本来の活性を有するものと考えられる。

【００４３】

【表６】

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(N末端側)TCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCA *Not I* 開始コドン
AAGTTTTTTTCTTCCATTTCAGGTGTCGTGAACACGTGGTC*GCGGCCGC*GTG**ATG**GCTCT
AATCCCAGACTTGGCCATGGAAACCTGGCTTCTCCTGGCTGTCAGCCTGGTGCTCCTCTA
TCTATATGGAACCCATTCACATGGACTTTTTAAGAAGCTTGGAATTCCAGGGCCCACACC
TCTGCCTTTTTTGGGAAATATTTTGTCCTACCATAAGGGCTTTTGTATGTTTGACATGGA
ATGTCATAAAAAGTATGGAAAAGTGTGGGGCTTTTATGATGGTCAACAGCCTGTGCTGGC
TATCACAGATCCTGACATGATCAAAACAGTGCTAGTGAAAGAATGTTATTCTGTCTTCAC
AAACCGGAGGCCTTTTGGTCCAGTGGGATTTATGAAAAGTGCCATCTCTATAGCTGAGGA
TGAAGAATGGAAGAGATTACGATCATTGCTGTCTCCAACCTTCACCAGTGGAAAACTCAA
GGAGATGGTCCCTATCATTGCCCAGTATGGAGATGTGTTGGTGAGAAATCTGAGGCGGGA
AGCAGAGACAGGCAAGCCTGTCACCTTGAAAGACGTCTTTGGGGCCTACAGCATGGATGT
GATCACTAGCACATCATTTGGAGTGAACATCGACTCTCTCAACAATCCACAAGACCCCTT
TGTGGAAAACACCAAGAAGCTTTTAAGATTTGATTTTTTGGATCCATTCTTTCTCTCAAT
AACAGTCTTTCCATTCCTCATCCCAATTCTTGAAGTATTAAATATCTGTGTGTTTCCAAG
AGAAGTTACAAATTTTTTAAGAAAATCTGTAAAAAGGATGAAAGAAAGTCGCCTCGAAGA
TACACAAAAGCACCGAGTGGATTTCCTTCAGCTGATGATTGACTCTCAGAATTCAAAAGA
AACTGAGTCCCACAAAGCTCTGTCCGATCTGGAGCTCGTGGCCCAATCAATTATCTTTAT
TTTTGCTGGCTATGAAACCACGAGCAGTGTTCTCTCCTTCATTATGTATGAACTGGCCAC
TCACCCTGATGTCCAGCAGAAACTGCAGGAGGAAATTGATGCAGTTTTACCCAATAAGGC
ACCACCCACCTATGATACTGTGCTACAGATGGAGTATCTTGACATGGTGGTGAATGAAAC
GCTCAGATTATTCCCAATTGCTATGAGACTTGAGAGGGTCTGCAAAAAAGATGTTGAGAT
CAATGGGATGTTCATTCCCAAAGGGTGGGTGGTGATGATTCCAAGCTATGCTCTTCACCG
TGACCCAAAGTACTGGAGAGAGCCTGAGAAGTTCCTCCCTGAAAGATTCAGCAAGAAGAA
CAAGGACAACATAGATCCTTACATATACACACCCTTTGGAAGTGGACCCAGAAACTGCAT
TGGCATGAGGTTTGCTCTCATGAACATGAAACTTGCTCTAATCAGAGTCCTTCAGAACTT
CTCCTTCAAACCTTGTAAAGAAACACAGATCCCCCTGAAATTAAGCTTAGGAGGACTTCT
TCAACCAGAAAAACCCGTTGTTCTAAAGGTTGAGTCAAGGGATGGCACCG**TAA**GTGGAGC
終止コドン
BglII
CTGAAT*AGATCT*GGCCGGCTGGGCCCGTTTCGAAGGTAAGCCTATCCCTAACCCTCTCCT
CGGTCTCGATTCTACGCGTACC

【００４４】又、P450 3A4 発現ベクターに挿入されたGS 遺伝子のシークエンス結果を以下の表７に示す。GS 遺伝子については、template としたpBK－CMV－GS のGS 遺伝子の配列30) と全く同じであった。pBK－CMV－GS を導入したCHO 細胞（CN9－500－4 細胞株）及びHepG2 細胞（HNAA－300A 細胞株）においてGS 活性が確認されていることより、このベクターに挿入したGS も本来の蛋白質としての機能を有していると考えられる。

【００４５】

【表７】

(N末端側)TCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATA
GGGAGACCC*AAGCTT*ACC**ATG**GCCACCTCAGCAAGTTCCCACTTGAACAAAAACATCAAG
HindIII 開始コドン
CAAATGTACCTGTGCCTGCCCCAGGGTGAGAAAGTCCAAGCCATGTATATCTGGGTTGAT
GGTACTGGAGAAGGACTGCGCTGCAAAACCCGCACCCTGGACTGTGAGCCCAAGTGTGTA
GAAGAGTTACCTGAGTGGAATTTTGATGGCTCTAGTACCTTTCAGTCTGAGGGCTCCAAC
AGTGACATGTATCTCAGCCCTGTTGCCATGTTTCGGGACCCCTTCCGCAGAGATCCCAAC
AAGCTGGTGTTCTGTGAAGTTTTCAAGTACAACCGGAAGCCTGCAGAGACCAATTTAAGG
CACTCGTGTAAACGGATAATGGACATGGTGAGCAACCAGCACCCCTGGTTTGGAATGGAA
GAGGAGTATACTCTGATGGGAACAGATGGGCACCCTTTTGGTTGGCCTTCCAATGGCTTT
CCTGGGCCCCAAGGTCCGTATTACTGTGGTGTGGGCGCAGACAAAGCCTATGGCAGGGAT
ATCGTGGAGGCTCACTACCGCGCCTGCTTGTATGCTGGGGTCAAGATTACAGGAACAAAT
GCTGAGGTCATGCCTGCCCAGTGGGAATTCCAAATAGGACCCTGTGAAGGAATCCGCATG
GGAGATCATCTCTGGGTGGCCCGTTTCATCTTGCATCGAGTATGTGAAGACTTTGGGGTA
ATAGCAACCTTTGACCCCAAGCCCATTCCTGGGAACTGGAATGGTGCAGGCTGCCATACC
AACTTTAGCACCAAGGCCATGCGGGAGGAGAATGGTCTGAAGCACATCGAGGAGGCCATC
GAGAAACTAAGCAAGCGGCACCGGTACCACATTCGAGCCTACGATCCCAAGGGGGGCCTG
GACAATGCCCGTCGTCTGACTGGGTTCCACGAAACGTCCAACATCAACGACTTTTCTGCT
GGTGTCGCCAATCGCAGTGCCAGCATCCGCATTCCCCGGACTGTCGGCCAGGAGAAGAAA
GGTTACTTTGAAGACCGCCGCCCCTCTGCCAATTGTGACCCCTTTGCAGTGACAGAAGCC
ATCGTCCGCACATGCCTTCTCAATGAGACTGGCGACGAGCCCTTCCAATACAAAAACT**AA**
TT*GGATCC*GAACAAAAACTCATCTCAGAAGAGGATCTGAATATGCA (C末端側) 終止コドン
BamH I

【００４６】

【実施例４】６．P450 3A4 発現ベクターの動物細胞への導入

P450 3A4 発現ベクターを野生株HepG2 細胞（ATCC No.

HB－8065)、及び本発明者により既に構築した遺伝子組換えアンモニア代謝HepG2 細胞（HNAA－300A株）に導入することを試みた。動物細胞にP450 3A4 発現ベクターを導入するにあたり、このベクターを高純度で調製しなくてはならない。そこで、Wizard PureFection Plasmid DNA Purification System （A2160 ;エンドトキシン等を除いた後、マグネットにDNA を吸着させて高純度のプラスミドDNA を回収する方法)を用いた。方法は、付属マニュアルに従った(方法の記載は略)。上記で得られた高純度のP450 3A4 発現ベクターを用いてtransfection を行った。transfection 方法はリポソーム法（TaKaRa, Trans IT Polyamine Transfection Reagents ）を用いた。

1)4 ×10 ^{5} 個の細胞を35 mm dish に播種、37 ℃で一晩培養した。培地としては、HepG2 ・CHO－K1 においてはFBS 入りRDF(Gln +)培地を、HNAA－300A ・CN9－500－4 においては透析血清入りRDF(Gln －)培地を用いた。

2)翌日、100 μ l の無血清培地にTrans IT Transfection Reagent を20 μ l 加え、vortex にて良く混合した。

3)室温にて10 min 、培養した。

4)これにベクターDNA 3 μ g を加え、穏やかにpipetting した。

5)室温にて10 min 、培養した。

6)5)で調製したものを、1)で準備した細胞培地にポタポタと添加した。dish をゆっくりと揺すって混合させた。

7)72 時間、37 ℃で培養した。

8)7)で調製したものを、トリプシン処理して剥がし全量を小－T フラスコ（底面積25cm 2 ）に播種した。培地はZeocin を含まないRDF(Gln + 及びGln －)培地を用いて、継代した。

9)小－T フラスコにおいてコンフルエントになった後、Zeocin を含む選択培地に植え継いだ。選択培地中のZeocin 濃度は、HepG2 及びHNAA－300A では200 μg/ml とした。

【００４７】こうして得られたP4503A4 を発現させたHepG2 細胞（CYP3A4－GS－HepG2）のP450 3A4 活性を測定した結果を表８に示す。尚、表中では、該細胞は、「P450 3A4－HepG2」と表記されている（以下、本明細書の各表中で同様）。

【００４８】

【表８】

細胞株	P450 3A4 活性 (testosterone 6β－hydroxylation 活性, pmol/min/mg-protein)
P450 3A4-HepG2	490
HepG2(薬物誘導なし)	0.6
HepG2(薬物誘導あり)	2.3

【００４９】表８から明らかなように、P450 3A4 導入HepG2 細胞における活性値は野生株の約800 倍高い値、490 pmol/min/mg－protein を示した。P450 3A4 を発現させたCHO 細胞（P450 3A4－CHO 細胞株）における活性と比較しても、P4503A4－ HepG2 細胞株における活性は約20 倍高い値を示したことになる。P450 3A4 導入CHO 細胞と比べ、P450 3A4 導入HepG2 細胞におけるP450 3A4 活性が20 倍以上も高くなったが、これはCHO 細胞に比べHepG2 細胞においてP450 の反応機構をサポートする環境（例えばP450 による酸化・還元反応において、P450 還元酵素からのP450 への電子供給が活発に行われているなど）が細胞内に残存しているためではないかと考えられる。

【００５０】ここで24 時間培養した初代肝細胞のP450 3A4 活性値は、252.8 pmol/min/mg－protein と報告されている35) 。またラット初代肝細胞を用いたものでは、4時間培養後のP450 3A4 活性が407pmol/min/mg－protein 、24 時間培養後の活性は158 pmol/min/mg－protein となっている36) 。このことよりP450 3A4 導入HepG2 細胞株は、初代肝細胞と同等もしくはそれ以上の活性を有していたと言える。しかしin vivo におけるヒト肝細胞のP450 3A4 活性は、個人差はあるものの1000 ～1500pmol/min/mg－protein 程度と言われており37) 、ヒトへの臨床応用に向けさらにP450 3A4－HepG2 細胞のP450 3A4 活性をさらに高めたいと考えた。

【００５１】

【実施例５】７．P450 3A4 発現動物細胞におけるGS 遺伝子系を用いた遺伝子増幅方法

P450 3A4 発現動物細胞におけるP450 3A4 活性を上昇させるために、GS 遺伝子系における遺伝子増幅を行った。

１） 小－T フラスコ（底面積25 cm 2 ）において、HepG2 細胞では200 μg/mlZeocin 及び各濃度のMSX を含む選択培地に細胞を約5 ×10 ^{5} 個播種し、コンフルエントになるまで培地交換を行った。

２） コンフルエントになれば細胞を剥離し、MSX （Methionine Sulfoximine ）濃度を上昇させた選択培地に約5 ×10^{5} 個播種するという行程を繰り返し、ヘテロな状態での遺伝子増幅を試みた。

【００５２】その結果、P450 3A4 導入HepG2 細胞において各MSX 濃度の耐性株を取得した。次にそれぞれの耐性株についてP450 3A4 活性を測定した(3)。その結果を

図３に示す。なおMSX がP450 3A4 活性に影響を及ぼしている可能性があったので、活性測定前の継代では培地にMSXを添加しなかった。また活性測定する際の培地にもMSX を添加しなかった。MSX 添加によりGS 遺伝子増幅系を利用したP450 3A4 活性の向上を目指したが、いずれの耐性株においてもほぼ同様な活性値を示した。このことより、GS 遺伝子における遺伝子増幅を用いてP450 3A4 活性を上昇させることはできなかったと言える。ここでなぜ各細胞株がMSX 耐性を取得したにも関わらず、P450 3A4活性が上昇しなかったのかについて以下のように考察した。

1)P450 3A4 発現ベクターに導入したGS 遺伝子によってではなく、内在性のGS遺伝子において遺伝子増幅が起こったので、P450 3A4 活性は上昇しなかった（この場合、P450 3A4 活性は変化なし、GS 活性は上昇することになる）。

2)P450 3A4 発現ベクターに導入したGS 遺伝子において遺伝子増幅が起こったが、何らかの要因でP450 3A4 遺伝子が増幅しなかった（この場合、P450 3A4 活性は変化なし、GS 活性は上昇する）。

3)GS 遺伝子系における遺伝子増幅そのものがうまくいっていなかった（この場合、P450 3A4 活性もGS活性も変化しない）。すなわち遺伝子増幅以外の機構により（例えば膜変異など）、MSX 耐性を獲得したと考えられる。

【００５３】

【実施例６】８． P450 3A4 導入HepG2 細胞におけるGS 活性測定法32)

これらのことを検証するために、各MSX 濃度耐性P450 3A4 導入HepG2 細胞のGS 活性を評価することにした。

８．１． GS 活性測定原理

GS は、γ－glutamyl 転移反応を触媒する。すなわち、hydroxylamine から、γ－glutamylhydroxyamate が生成し、この、γ－glutamylhydroxyamate が、ferric chloride の添加で特徴的な茶色を示す。これが、hydroxylamine を用いたglutamine synthetase の活性測定の原理である。この反応を利用し、GS 活性の測定を行った。

【００５４】８．２． GS 活性測定方法

1)細胞懸濁液をTBS で一度洗浄した後、500 μL のImidazole buffer に懸濁した。

2)10%β－mercaptoethanol を5 μL 加えた（(酸化防止剤)。

3)超音波菌体破壊装置(T－ A－ 4200;海上電機株式会社)により、10 秒間超音波処理後、1 分間 氷冷を３ 回繰り返すことにより、細胞を破砕した。

4)10%β－mercaptoethanol を再度、5 μL 加えた。

5)100 mM phenylmethylsulfonylfluoride (PMSF;エタノール溶液)を5 μL 加えた。

6)200 mM Pepstatin A (エタノール溶液)を5 μL 加えた。（PMSF とPepstatin A はプロテアーゼ阻害剤である）

7)18,000 ×g.で5 分間、4 ℃で遠心分離した。

8)上清を新しいエッペンに移し、体積を測定した。

9)粗酵素液に反応基質液を加え、ボルテックスした後、37 ℃で正確に15 分間反応させた。

10)0.75 mL のFeCl3 溶液を加え、18,000 ×g.で5 分間遠心分離した後、上清をキュベットにうつし、A 535 で吸光度を測定した。11)測定値からblank の値を引いて、活性を求めた。この測定条件下でA 535 =0.340 の時、1 unit とした32)。表９にγ－glutamyl transfer 活性測定試薬の組成を、表１０にはFeCl３ 溶液の組成を示した。

【００５５】

【表９】

	Stock液の組成	測定時における添加量
Imidazole-HCl	100 mM, pH7.2	250 μL
$MnCl_2$	125 mM	20 μL
L-Glutamine	250 mM	100 μL
arsenate	200 mM, pH7.2	50 μL
Hydroxylamine	1 M, pH7.2	50 μL
ADP･Na	2.5 mM	20 μL
酵素液		10 μL
Total volume		500 μL

【００５６】

【表１０】

	Stock液の濃度	測定時における添加量
$FeCl_3$	1.11 M	250 μL
HCl	2.01 M	250 μL
Trichloroacetic acid	0.60 M	250 μL
Total volume		750 μL

【００５７】また、GS 活性を測定することが目的である為、GS 酵素反応以外の吸光値は、除かれるべきである。そこで、表１１に示すようなblank をとった。

【００５８】

【表１１】

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	b_1	b_2	b_3
Imidazole-HCl	○	○	○
$MnCl_2$	○	○	○
L-Glutamine	×	○	×
arcenate	○	○	○
Hydroxylamine	×	○	×
ADP·Na	×	○	×
酵素液	○	×	×

【００５９】注）尚、ｂ１、ｂ２、ｂ３はblank の種類を示す。また、上記表の○は添加、×は添加しないことを示す。ｂ１は基質を抜いたもの、ｂ２は酵素を抜いたもの、ｂ３は基質と酵素を抜いたものである。液量は、超純水を添加して500 μL にした。最終的に酵素活性測定時におけるblank は、以下の様にして計算した。

blank =(b 1)＋(b 2)－(b 3)

【００６０】図４に合成培地を用いた培養におけるP450 3A4 導入HepG2 細胞（各MSX 濃度耐性株）のGS 活性（合成培地中）の結果を示す。各MSX 濃度耐性P450 3A4 導入HepG2 細胞におけるGS 活性に、差は見出されなかった。このことより、各MSX濃度耐性株においてP450 3A4 活性に差がなかったのは、GS 遺伝子系における遺伝子増幅がうまく起こっていなかったためと考えられる。これら各MSX濃度耐性株は、遺伝子増幅以外の機構（例えば、膜変異など）により耐性を取得したと考えられる。今後、遺伝子増幅系を用いてP450 3A4 活性を上昇させることを考えるのであれば、GS 遺伝子以外の遺伝子増幅系（CAD 遺伝子系など）を用いなくてはならない。

【００６１】

【実施例７】９．血清でのP450 3A4 導入HepG2 細胞におけるP450 3A4 活性測定法30)

ところで臨床応用においては、キグナス（細胞培養装置）中でブタもしくはヒトの血漿が遺伝子組換え動物細胞と接することになる。そこで、以下の方法で、より応用に近い形として、P450 3A4 導入HepG2 細胞の薬物代謝能（P450 3A4 活性）を、合成培地ではなく、より血漿に近い成分である血清を用いた培養で評価することにした。また各MSX 濃度耐性株についても、同様に活性を測定した。

1)100 mm dish に細胞を1 ×10 ^{7} 個、播種。培地はRDF (Gln ＋)を用いた。培地量は10 mL 。5 %CO 2 、37 ℃のもと、16 時間培養した。

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2)培地を抜き取り、血清（FBS ）を10mL 加え、基質であるtestosterone を濃度が100 μ M になるように加えた。

3)5 %CO 2 、37 ℃のもと、2 時間培養。この培養上清2 mL を用いて、活性を測定した。

4)培養終了後、トリプシン処理により細胞を剥がし細胞総蛋白量を測定した。

【００６２】得られた結果を図５に示す。いずれの細胞株においても、P450 3A4 活性は200pmol/min/mg－protein 前後の値を示した。これは合成培地中で測定したP450 3A4 活性の約2/5 の値であった。しかしながらMSX 添加によるGS 遺伝子系遺伝子増幅のP450 3A4 活性における効果は見られなかった。

【００６３】

【実施例８】10．P450 3A4 導入HepG2 細胞におけるリドカイン代謝能の測定方法31)

これまでP450 3A4 活性を測定する上での基質として、ホルモン物質であるtestosterone （6 β位の水酸化）を用いた。P450 3A4 導入HepG2 細胞をバイオ人工肝として利用することを想定した際、testosterone 以外の薬物の代謝に関しても評価（確認）しておく必要がある。そこで、バイオ人工肝薬物代謝能を評価する際汎用されているリドカインの代謝能を測定することにした。リドカインは第一相反応のモデル基質として、また肝不全時の臨床指標として用いられている。

・HPLC 条件

HPLC 装置 …島津製作所製LC10AD 液体クロマトグラフシステム

使用カラム …C 18 カラムであるInertsil ODS－3V (4.6 ×150 mm,5 μ m,GL Sciences Inc.)

使 用buffer … 20 mM NaClO 4 ((pH 2.5)、15%acetonitrile (脱気を行った)

流 速… 2.0 ml/min

カラム温度 …室温

測 定波長 … 205 nm

【００６４】その結果を表１２に示す。この表からわかるようにP450 3A4 導入HepG2 細胞におけるリドカイン代謝能は、5.1 nmol/min/mg－protein であった。尚、値は2回測定平均値であり、各測定値は4.7 、5.5 。

【００６５】

【表１２】

細胞株	P450 活性 (lidocaine 3- hydroxylation 活性, nmol/min/mg-protein)
P450 3A4-HepG2	5.1
HepG2	<0.1

【００６６】

【実施例９】11．長期間にわたる薬物代謝活性の維持

更に、長い期間に亘って、本発明の細胞株が活性を維持出来るか否かを検討した。その為に、本発明の細胞株で

あるCYP3A4及びＧＳを組み込んだＨｅｐＧ２を８０日間以上（約２０世代以上）培養し、その薬物代謝活性について測定した。得られた結果を以下の表１３に示す。その結果、蛋白質mg当たり約４２６pmol/minという高い活性が維持されることが判明した。

【００６７】

【表１３】

Table GS-P450 3A4 発現ベクター導入の効果

	P450 3A4 活性 (testosterone 6β-hydroxylation activity) (pmol min^{-1} $mg\text{-}protein^{-1}$)
CHO-K1 細胞(野生株)	< 0.1
P450 3A4 導入 CHO 細胞	21
HepG2 細胞（野生株）	0.6
P450 3A4 導入 HepG2 細胞	490
P450 3A4 導入 HepG2 細胞(100%血清培養)	200
P450 3A4 導入 HepG2 細胞(80 日間以上、約 40 世代継代後)	426
ヒト初代肝細胞(生体より単離 24 時間後)	250*
ヒト初代肝細胞(生体より単離 96 時間後)	11**
ヒト生体肝	1000～1500***

*Journal of Hepatology 31:549 (1999)
**BiochemicalSocietyTransactions 22:131S (1994))
***Biochemical Pharmacology 40:2525 (1990)

【００６８】

【実施例１０】12．クリアランスに基づくバイオ人工肝としての薬物代謝能の評価

12．1 クリアランス計算の理論

ここでP450 3A4 導入HepG2 細胞のリドカイン代謝能を基に、この細胞をキグナス（細胞培養装置）に適用した際のバイオ人工肝としての薬物代謝能を評価することにした。またこの評価にあたり、指標としてクリアランスを用いた38),39),40)。一般的にクリアランスは、臓器が薬物を処理する能力の大きさを示す。ここでいう処理は、代謝のみならず薬物の消失や生体膜を透過する移動など全てを含めた考え方である。クリアランス(CL)（ml/min)は、血液中の濃度(C)（mg/ml）の薬物が速度Vel（mg/min）で処理される時、次式で定義される。

Vel =CL ・C

ここで、バイオ人工肝に流入する血液中（血漿中）薬物濃度C in（mg/ml）を基準とするクリアランスをバイオ人工肝クリアランス（CL人工肝 ）（ml/min）と定義すると、下式で表される。

Vel =CL 人工肝 ・C in

【００６９】12．２．クリアランスの計算

ここでバイオ人工肝に薬物濃度C in（mg/ml）の血漿が流速Q (ml/min)で流入し、P450 3A4 導入HepG2 細胞による代謝により薬物濃度がC out（mg/ml）と小さくなって流出するモデルを考えると、薬物の流入速度V in（mg/min）と流出速度V out（mg/min）、バイオ人工肝内処理速度Vel（mg/min）はそれぞれ薬物

の流入速度 ： V in =Q ・C in

薬物の流出速度 ：V out =Q ・C out

バイオ人工肝内処理速度 ：Vel =CL 人工肝 ・C in

と表される。これよりバイオ人工肝内の薬物量変化速度(V)（mg/min）は、

V = Vin － Vout－Vel

=Q(C in －C out)－CL 人工肝・C in ……①

と表される。ここで定常状態では、バイオ人工肝内での薬物濃度変化はないので、V =0 となり

CL 人工肝 ＝Q・(C in－ C out)/C in

となる。

ここで (C in － C out)/C in＝E r ……②

とおくと、

CL 人工肝 ＝Q・E r ……③

となる。Er を抽出効率と呼び、これは血漿がバイオ人工肝を通過する間に、薬物が処理される割合を表す。

【００７０】12．３．バイオ人工肝クリアランスと固有クリアランスの関係

バイオ人工肝クリアランス（CL 人工肝）を求める際に、固有クリアランス（CL int ）について考えなくてはならない。まず固有クリアランスとは、バイオ人工肝中の薬物が消失する微小部位の有効薬物濃度（Ce ）を基準にしたものであり、バイオ人工肝が本来所有する処理能力、いわばP450 3A4 導入HepG2 細胞における処理能力を表すと考えればよい。ここで仮定として、血漿中の血漿蛋白などと結合していない非結合型薬物濃度（ f ・C ）が、バイオ人工肝内の処理部位の非結合型薬物濃度と濃度平衡の関係にあり、非結合型薬物だけが処理されるとする。ここで、f は薬物の血漿蛋白との結合率を表す。バイオ人工肝内有効薬物濃度(Ce)は均一で、流出する血漿中非結合型濃度（ f・C out ）に等しいと考えると、

f ・C out＝C e

より

Vel =dn/dt =CL 人工肝 ・C in =CL int ・Ce

=CL int ・f ・C out ……④

となる。

【００７１】12．４．バイオ人工肝クリアランス
定常状態では、①式においてV =0 となることより、④式より
Q(C in−C out)=CL int ・f ・C out となる。
この式と②式より、抽出効率Er は
Er =f ・CL int /(Q +f ・CL int)
となり、③式より
CL 人工肝 =Q ・f ・CL int /(Q +f ・CL int)……⑤
となる。

【００７２】12．５．固有クリアランス
P450 3A4 導入HepG2 細胞による薬物代謝が、ミカエリスーメンテンの式に従うと仮定すると、代謝部位の基質濃度、すなわちバイオ人工肝内有効薬物濃度をCe 、Ce無限大での薬物の最大代謝速度をV max 、ミカエリス定数をK m とすると、
Vel =V max ・Ce/(K m +Ce)
となる。また固有クリアランスは④式で表されることより、
CL int =V max /(K m +Ce)……⑥
と表される。

【００７３】12．６．バイオ人工肝クリアランスの試算
例えば、特開平06−113818号公報に開示されているような、キグナス（細胞培養装置：細胞数約4 ×10^9 cells 、容積1 L ）41),42) にP450 3A4 導入HepG2細胞を充填したと仮定して、バイオ人工肝クリアランスを試算した。既に測定したP450 3A4 導入HepG2細胞におけるリドカイン代謝能5.1 nmol/min/mg−protein、リドカイン代謝におけるK m =65 μM （文献値引用）43) 及びバイオ人工肝内有効リドカイン濃度すなわち定常状態における血液中のリドカイン平均濃度Ce=25 μM 43) を基に、⑥式よりCL int の値を算出した。その結果、CL int =57ml/min と求まった。この値と、キグナス（細胞培養装置）における血流速度Q=15 ml/min 41) 及びリドカインの血漿蛋白との結合率（文献値）f =0.3 43)を基に、バイオ人工肝クリアランスを試算すると、CL 臓器 = 8.0 ml/min と求まった。これはラット初代肝細胞を用いたバイオ人工肝（CL =7.0 ml/min ）44)とほぼ同等の値を示した。従ってP450 3A4 導入HepG2 細胞を用いたバイオ人工肝は、薬物代謝において初代肝細胞を用いたバイオ人工肝と同じオーダーの機能を有することがわかった。初代肝細胞は生体からの調達が必要であり、肝機能を長期にわたり維持することが困難なこと・ウイルス感染の危険性があることなどを考慮すると、無限増殖能を有するP450 3A4 導入HepG2 細胞は、バイオ人工肝に用いる細胞として有用であろう。ただしバイオ人工肝クリアランスを算出するにあたり、細胞培養装置内に充填する細胞自体が有する機能のみならず、バイオ人工肝に流入する血流速度に大きく依存する事に留意しなくてはならない。例えばヒト肝における肝クリアランスは約70 0 ml/min 45）である。これは先に試算したバイオ人工肝の約100倍の値であるが、ヒト肝における血流速度は約1600 ml/min 40) であり、バイオ人工肝（キグナス：15ml/min ）の血流速度の約100 倍である。

【００７４】13. 人工肝補助実験
実際に上記の装置及び培養条件において、本発明の細胞株である CYP3A4−GS−HepG2を用いて、人工肝補助実験を実施した。図６には実際の肝不全モデルを示し、図７には人工肝補助システムの評価に用いた有効生存時間の定義を示した。図８は、各実験群における有効生存時間の結果を示す。ここで、「無処置」は何もほどかさない群、「無細胞人肝」は細胞のない状態の人工肝システムを接続して循環させたもの、「血漿交換＋ＣＨＤＦ（持続的血液濾過）」は一般的に肝不全の治療に用いられる方法、「ＧＳ－ＨｅｐＧ２」は、本発明の細胞株を使用した群、及び「Ｗｔ－ＨｅｐＧ２」は、野生株（遺伝子を導入する前の細胞株）を使用した群である。図８に示された結果から、本発明の肝機能補助装置により、優れた延命効果が得られたことが判る。更に、図９及び図１０に示されるように、脳圧亢進発現頻度、並びに、血液凝固系におけるＡＰＴＴ活性部分トロンボスラスチン活性、ＡＣＴ活性凝固時間、ＨＰＴヘパプラスチン時間、及びＦＶＩＩ第７因子といった各種パラメーターにおいて優れた改善効果が認められた。

【００７５】
【実施例１１】本発明細胞株（ＣＹＰ３Ａ４－ＧＳ－ＨｅｐＧ２）を使用した薬物代謝アッセイ系
細胞にニフェジピンを添加し、経時的に細胞外液のニフェジピンおよび水酸化体濃度を測定した。１００ｎＭでは添加５分後には添加量の５％相当量の水酸化体が検出可能となり、３０分では５０％まで直線的に増加した。また、６０分では７５％となり、この時点では親薬物は検出感度以下となった。一方対象とした細胞では６０分でも平均７％であった。（図１１）この系に３Ａ４阻害薬である１００μＭのケトコナゾール、シメチジン、エリスロマイシンを１０分間前処理後にニフェジピンを加えると、３０分後の水酸化体検出量は非処理時のそれぞれ２５－３５％となった。（図１２）一方、ニフェジピンの代わりにミダゾラムを用いて水酸化体測定を行っても、ケトコナゾール、シメチジン、エリスロマイシン前処置により、水酸化体検出量は非添加時の３５－４５％となった。（図１３）。よってこの細胞に未知薬物を添加し、ニフェジピン、ミダゾラムの水酸化を測定することで、その薬物に３Ａ４阻害効果があるか否かを簡便に評価することが可能である。特に本細胞では、培養液中へ流出した水酸化体が測定可能であるほど、代謝能が高いことが特徴である。

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【図面の簡単な説明】

【図１】野生株ＨｅｐＧ２細胞に対するヒト肝薬物代謝酵素誘導薬rifampicin添加培養実験で得られた結果を示す。

【図２】P450 3A4 遺伝子及びGS 遺伝子のPCR産物の0.8%アガロースゲル電気泳動により得られた写真を示す。

【図３】P450 3A4 導入HepG2 細胞（各MSX 濃度の耐性株）におけるP450 3A4 活性（合成培地中）を示す。

【図４】P450 3A4 導入ＨｅｐＧ２細胞（各MSX 濃度耐性株）のGS 活性（合成培地中）の結果を示す。

【図５】血清でのP450 3A4 導入ＨｅｐＧ２細胞におけるP450 3A4 活性測定の結果を示す。

【図６】実際の肝不全モデルを示す。

【図７】人工肝補助システムの評価に用いた有効生存時間の定義を示す。

【図８】各実験群における有効生存時間の結果を示す。

【図９】脳圧亢進発現頻度の結果を示す。

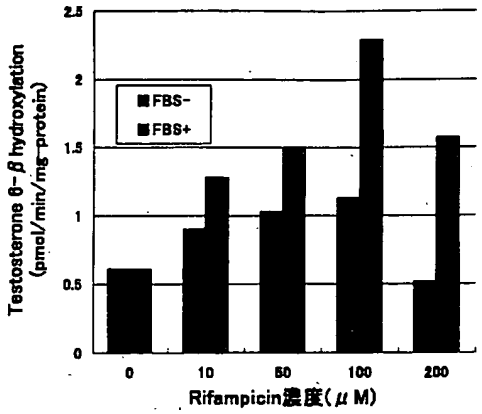
【図１０】血液凝固系における各種パラメーターに関する結果を示す。

【図１１】本発明細胞株によるニフェジピンの代謝の様子を示す。

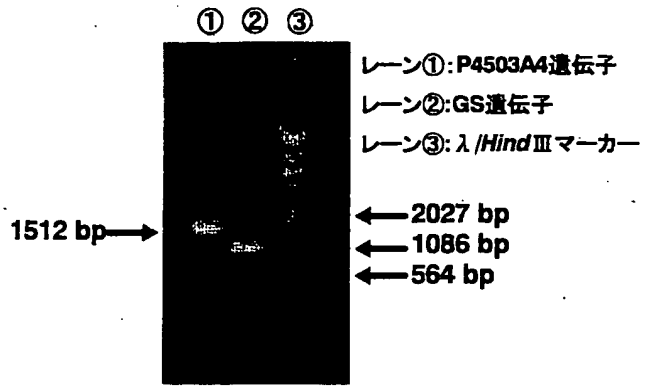
【図１２】ニフェジピン代謝に対する各阻害薬の影響を示す。

【図１３】ミダゾラム代謝に対する各阻害薬の影響を示す。

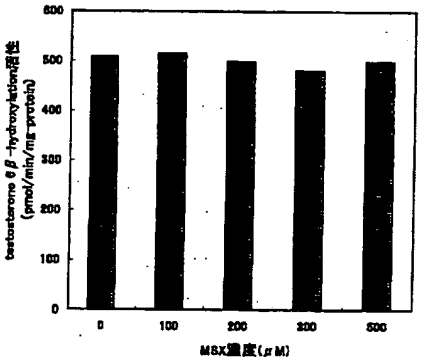
【図１】



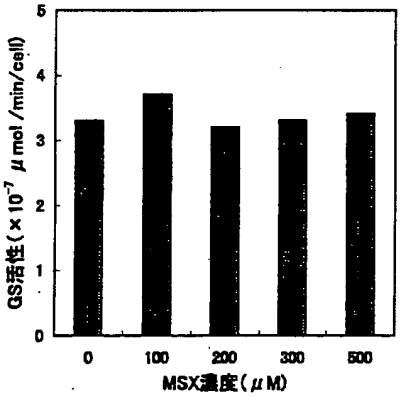
【図２】



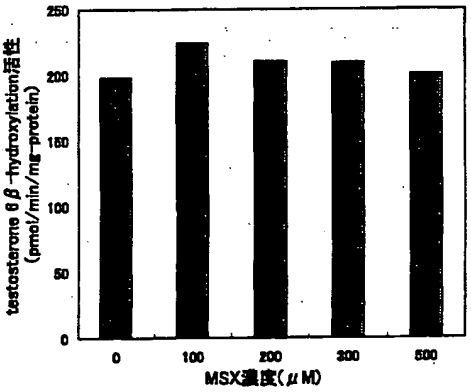
【図３】



【図４】

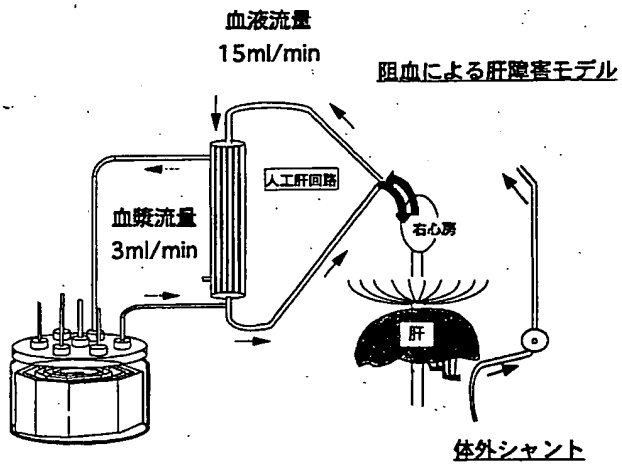


【図５】



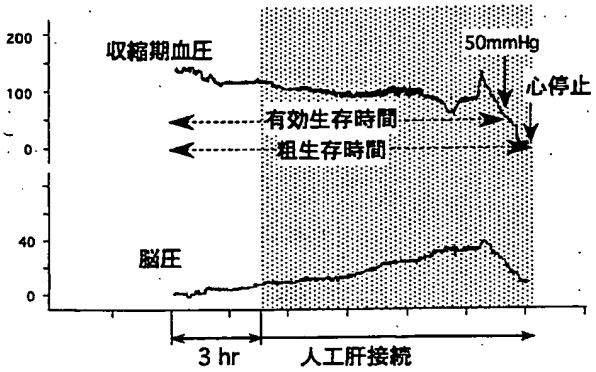
【図６】

回流式培養装置（キグナス）を用いた
ハイブリッド人工肝



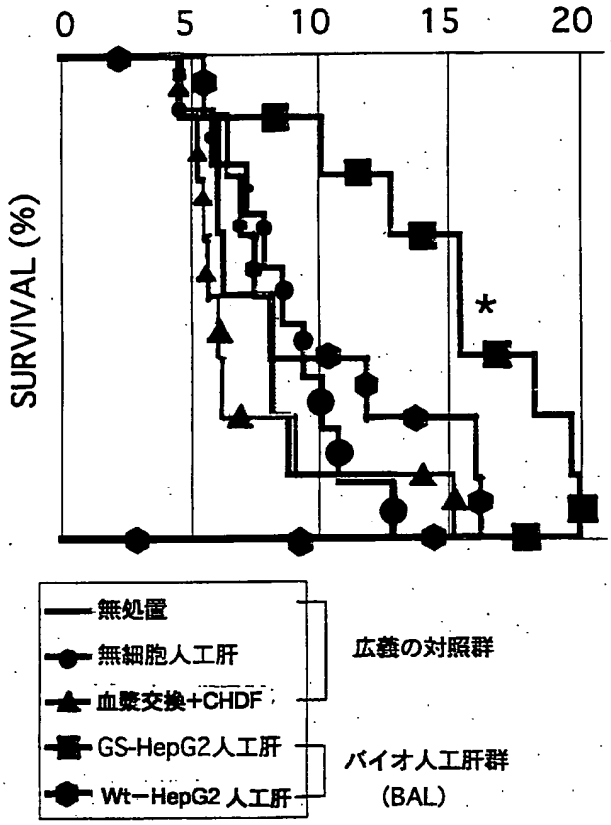
【図７】

阻血性肝不全ブタの血圧・脳圧の推移と粗生存時間、有効生存時間



【図８】

各実験群の有効生存時間の比較

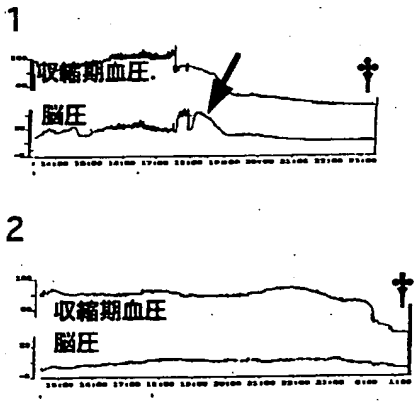


【図９】

BAL処置群と他群における脳圧亢進発現頻度の比較

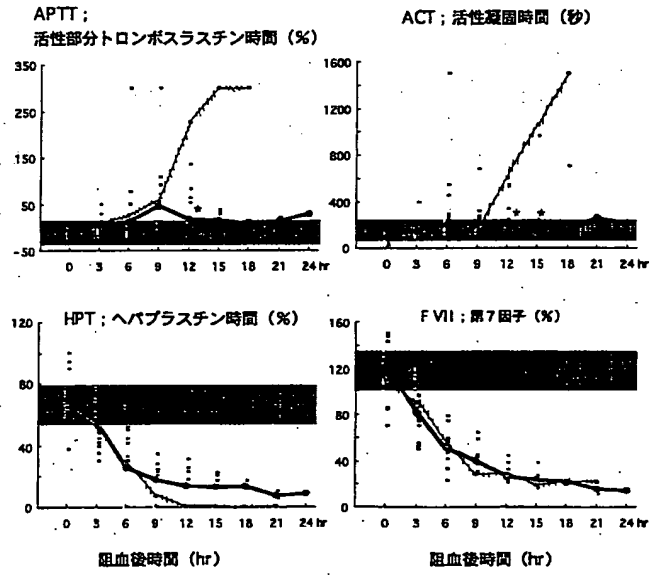
脳圧	HepG2BAL	他群
亢進例	2	11
非亢進例	14	14

カイ２乗検定, p=0.0345



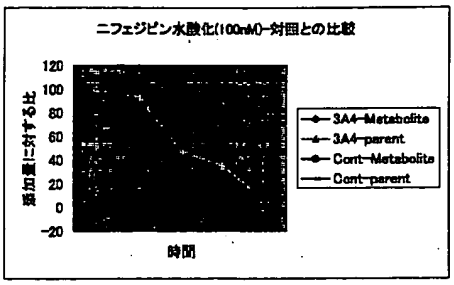
【図１０】

BAL処置有(−) 無 (┅)で比較した場合の血液凝固系の推移 （中央値）



【図１１】

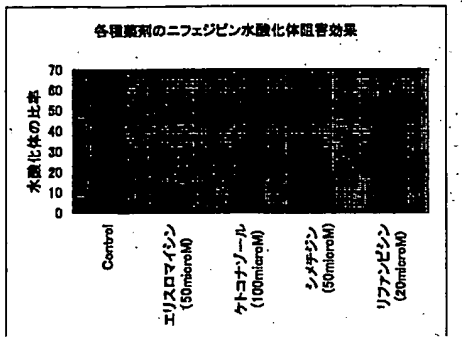
	3A4				Control			
Time(min)	Metabolite	SD	Parent	SD	Metabolite	SD	Parent	SD
0min	0		100		0		100	
5min	9	2	83	3	0		99	1
10min	20	4	48	5	0		98	2
30min	52	5	35	8	2	2	94	4
60min	77	7	7	8	7	4	92	4



【図１２】

ニフェジピン(100nM)30分添加時水酸化体産生に対する阻害薬の影響
総添加量を100%としたとき

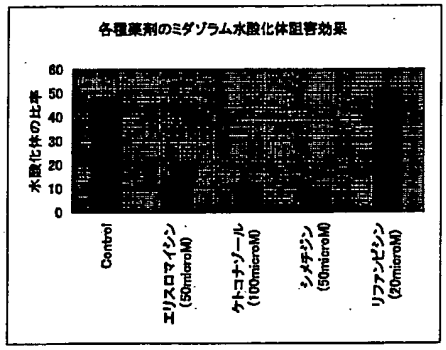
	%	SD
Control	52	5
エリスロマイシン(50n	13	4
ケトコナゾール(100n	17	4
シメチジン(50microM	19	6
リファンピシン(20mic	55	7



【図１３】

ミダゾラム(250nM)30分添加時水酸化体産生に対する阻害薬の影響
総添加量を100%としたとき

	%	SD
Control	48	6
エリスロマイシン(50n	15	5
ケトコナゾール(100n	12	3
シメチジン(50microM	10	4
リファンピシン(20mic	52	5



フロントページの続き

(51) Int.Cl.[7]		識別記号	FI		テーマコード(参考)
A61P	1/16		A61P	43/00	101
	7/08				105
	43/00	101	C12N	15/00	ZNAA
		105		5/00	B

(72)発明者　岸本　通雅
京都府京都市西京区桂原角田町１－24
(72)発明者　片倉　啓雄
大阪府箕面市小野原東４－15－16
(72)発明者　絵野沢　伸
東京都北区浮間４－26　浮間住宅３－205
(72)発明者　鶴岡　秀一
栃木県河内郡南河内町祇園一丁目９番４号

Ｆターム(参考) 4B024 AA01 AA11 BA08 BA11 DA03
EA04 FA20 GA11 GA18 GA30
HA20
4B063 QA05 QA18 QA19 QQ08 QQ61
QQ91 QR02 QR20 QR77 QS24
QS39 QX01
4B065 AA93X AB01 AC20 BA02
BB40 CA27 CA28 CA44 CA46
4C077 AA07 BB10 EE01 PP29
4C087 AA02 BB52 BB64 BB65 DA04
DA08 NA05 NA10 ZA51 ZA75
ZC37